Antitumor Activities of Polysaccharides from the Long-veiled Lady Mushroom *Dictyophora indusiata*

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Abstract

In "Shen Nong's Herbal" of the East Han dynasty in China, the beneficial effects of various fungi were already illustrated. In the last three decades, many studies have focused on the antitumor and immunomodulatory effects of mushroom polysaccharides and exploited their potential as clinical anticancer drug or adjuvant. In this project, the famous edible long-veiled lady mushroom *Dictyophora indusiata* was selected for both *in vivo* and *in vitro* antitumor studies of its polysaccharides, which have received only little attention in research.

Three DI3 polysaccharides fractions were extracted from *Dictyophora indusiata*, and designated as DI3a, DI3b and DI3c, respectively, in present study. They were isolated by water extraction and ethanol precipitation, followed by anion-exchange fractionation. DI3c fraction is relatively homogenous protein-containing polysaccharide with molecular weight of 3.3 kDa, while the fractions DI3b and DI3c contained more than one group of polysaccharides. Using *in vivo* antitumor study, DI3c exhibited the most prominent antitumor activity in Sarcoma 180 tumor-bearing BALB/c mice. Administration of 50 mg/kg of DI3c fraction for 10 consecutive days (i.p.) could inhibit the growth of Sarcoma 180 solid tumor by 76%. In the study of cytokine production in blood serum, DI3c fraction not only induced the production

of TNF-α, but also primed the LPS-triggered TNF-α production at a higher level. However, no up-regulation of IL-2 was observed. DI3c did not inhibit the growth of S-180 cell *in vitro*, and hence, the anti-tumor activity of DI3c is thought to be due to the immunomodulatory effect via TNF-α production at least partially. On the other hand, *in vitro* studies showed that, DI3b fraction could inhibit the proliferation of HL-60 and HepG2 cells significantly in a dose-dependent manner. At the dose of 200 μg/ml, DI3b could give 50% inhibition rate on proliferation of HL-60 cells after 72 h incubation. The dose-dependent antiproliferative effect of DI3b fraction on HL-60 cells was verified by the BrdU incorporation as well.

摘要

在過去的三十年，真菌多糖對於抗腫瘤的作用已廣被研究。而遠於東漢年代，菌類在醫學上的療效更已載於《神農本草經》。近年來，真菌多糖亦被發展成為抗癌或抗癌的補助藥品。進一步了解及研究菌類多糖對抗癌的藥用價值，食用菌長裙竹蓀被選為本研究的樣本，提純此菌類的各種多糖，並對其抗癌作用進行體內及體外的詳細實驗。

本研究於竹蓀中提取了多糖 DI2 和 DI3，並於 DI3 多糖中再萃提出三個多糖部份，分別命名為 DI3a、DI3b 及 DI3c。在三個 DI3 多糖部份當中，DI3c 是較純化多糖蛋白部份，分子量約為 3.3 kDa。而 DI3a 及 DI3b 則由多於一組的多糖組成。

在體內抗腫瘤實驗顯示，竹蓀多糖 DI3b 與 DI3c 均對小鼠肉瘤 S-180 的生長有一定的抑制作用，而比較這兩個竹蓀多糖部份，DI3c 是最為有效的。DI3c (50mg/kg × 10 天)能有效地抑制小鼠肉瘤 S-180 的生長達百份之七十六。

另外，實驗結果顯示 DI3c 可以提升 BALB/c 小鼠的免疫能力。多糖 DI3c 能提升小鼠血清的腫瘤壞死因子 (TNF-α) 濃度。而 DI3c 對提升 TNF-α 的製造能力，於注射脂多糖 (LPS) 後更為顯著。相

反，DI3c 對白介素-2 (IL-2) 的製造並無任何作用。此項實驗顯示 DI3c 的抗腫瘤作用與 TNF-α 的製造有直接的關係。

於體外抗腫瘤實驗中，多糖 DI3b 部份對白血細胞 HL-60 及肝癌細胞 HepG2，均具有抑制增殖作用，並表現出劑量依賴性抑制。經過三天體外培養，DI3b (200 μg/ml) 能有效抑制百份之五十的 HL-60 細胞增殖。而通過溴脫氧尿苷合併實驗(BrdU)，更進一步證實 DI3b 於抑制癌細胞增殖的作用。

Table of Contents

List of Tables

List of Figures

List of Figures (continued)

List of Abbreviations

ATCC	American type culture collection
AZT	Azidothymidine
BCA	Bicinchoninic acid assay
BrdU	5-bromo-2'-deoxyurdine
Cdk	Cyclin dependent kinase
CPE	Cytopathic effect
DEAE-cellulose	Diethylaminoethyl cellulose
DI	*Dictyophora indusiata*
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GC	Gas chromatography
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HSV	Herpes simplex virus
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IL	Interleukin
LDH	Lactate dehydrogenase

List of Abbreviations (continued)

LPS	Lipopolysaccharides
M-CSF	Macrophage-colony stimulating factor
MEM	Minimal Essential Medium
MTT	3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	Phosphate buffered saline
RB	Retinoblastoma
RT-PCR	Reverse-transcriptase polymerase chain reaction
s.c.	Subcutaneous
S.D.	Standard deviation
SD	Sprague Dawley
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumor necrosis factor

Chapter 1

Introduction

In the last three decades, it had been recognized that mushroom polysaccharides show a wide diversity of pharmacological activities, including antitumor, immunomodulatory, antiviral, hypoglycermic and free radical scavenging activities. Most of the studies are mainly focused on the antitumor and immunomodulatory effects of mushroom polysaccharides from the class of Basidomycetes (Wasser & Weis, 1999). Although, the chemotherapy has been established and widely used in clinical anticancer treatment, there is still an increase in significance in exploring mushroom polysaccharides with antitumor effects due to the dissatisfaction and occurrence of side effect by the current chemotherapy.

Among the mushroom polysaccharides that have been investigated in the last thirty years, they are mainly antitumor β-glucan, heteroglycan and polysaccharide protein complexes. Lentinan, which has been used in clinical anticancer treatment, is

a purified (1→6)-β-D-glucan isolated from *Lentinus edodes*. The research done by Chihara *et al.* (1970) had demonstrated the prominent antitumor effect of lentinan against Sarcoma 180 cells in Swiss albino mice and SWM/MS mice. After treated with a dose of 1mg/kg of lentinan for 10 consecutive days, complete regression of tumor was observed in tumor-bearing SWM/Ms mice (Chihara *et al.*, 1970). Besides, as suggested by Maeda & Chihara (1971), the antitumor effect of lentinan was shown to be contributed by the immune system, since the antitumor activity of lentinan was lost in neonatally thymectomized mice (Maede & Chihara, 1971).

Although the mechanism that mushroom polysaccharides produce their antitumor action is very complicated and shows differences between mushroom of different species, it seems that most of the immunomodulatory activities produced are correlated to the modulation of cytokine production in the host, for example the antitumor effect of lentinan is to up-regulate the IL-1α, IL-1β, TNF-α and IFN-γ in mice so as to enhance the immune system of the host to act against the tumor cells (Liu *et a*l., 1999). In order to study the cytokine expression induced by mushroom polysaccharides, different kinds of studies were applied, including Northern blot and reverse transcriptase polymerase chain reaction (RT-PCR). The immunomodulatory effects in cytokine production by the polysacchariopeptide (PSP) from *Coriolus versicolor* and polysaccharides-protein complex (PSPC) from *Tricholoma lobayense*

were analyzed by northern blot and RT-PCR, respectively, in different studies (Liu *et al.*, 1993; Liu *et al.*, 1999). Besides, by using indicator cells or enzyme-linked immunosorbent assay (ELISA), the cytokines present in biological fluid can also be measured.

Although most of the mushroom polysaccharides posses antitumor activity through immunomodulation, it may not be neglected the *in vitro* antiproliferative effect on cancer cells. In a recent *in vitro* study, a *Ganoderma lucidum* polysaccharide was found to exhibit antiproliferative effect on K-562 leukemic cells in a dose and time-dependent manner (Zhong *et al.*, 1999). Besides, restoration of cell cycle regulation was also observed in some other anticancer substances. One of the examples is ceramide, which produces antiproliferative effect on leukemic cells by acting as a key regulator in preventing progression through G1 to S phase in the cell cycle (Connor *et al.*, 2001). In order to investigate the antitumor effect produced by mushroom polysaccharides in detail, it is worth for us to perform the *in vitro* antitumor study in parallel to that of in animal model.

In the present study, edible long-veiled lady mushroom *Dictyophora indusiata*, which belongs to the class of Basidomycetes, was selected for investigation. It is a beautiful, phallic shaped mushroom and distributed mainly in China and Japan. In the previous studies, six different mushroom polysaccharides have been isolated

from *Dictyophora indusiata* (Hara *et al.*, 1982a, 1982b, 1983, 1986, 1988 & 1991; Ukai *et al.*, 1980, 1982 & 1983) and their antitumor effect was also studied by Ukai *et al.* (1983). However, the *in vitro* anticancer effects as well as the actions of the mushroom polysaccharides in producing antitumor activities have not been investigated in those studies. Polysaccharides from fruit bodies *Dictyophora indusiata* were isolated and fractionated in this study. Both *in vivo* and *in vitro* studies were performed to study the effectiveness of the polysaccharides fractions in inhibiting the growth of tumor. In order to study the mechanistic action of antitumor effect in the mouse model, enzyme-linked immunosorbent assay was also used in studying the cytokine production induced by the effective polysaccharides fractions.

Chapter 2

Literature Review

2.1 Mushroom Polysaccharides from Basidiomycetes

Mushroom has been exploited as food for thousands of years throughout the world especially in China and Japan. Both fresh and dried mushrooms are used as materials in Chinese, Japanese and Western cuisine, due to their excellent flavor and texture. Besides, it has been shown that most mushrooms have high nutritional value and may serve as a source of dietary protein, essential amino acids, fiber, vitamins and minerals. Furthermore, mushrooms have a high proportion of unsaturated fatty acid and a low content in calories, sodium, fat and cholesterol. Apart from the nutritional value, mushroom also has a long history in medicinal purpose. In "Shen Nong's Herbal" of the East Han dynasty in China (100-200AD), the beneficial effects of various fungi such as *Ganoderma lucidum*, *Poria cocos* and *Tremella fuciformis* were illustrated.

In the last three decades, the study of pharmacological activities of mushrooms in the class of Basidiomycetes has become matter of interest. Since, the polysaccharides extracted from Basidiomycetes have been served as bioactive components that express promising antitumor, immunomodulatory (Ooi and Liu, 2000), antiviral (Tsukagoshi *et al.*, 1984; Eo *et al.*, 1999), hypoglycermic (Hikino *et al.*, 1985), free radical scavenging (Liu *et al.*, 1996a) and other medicinal activities (Cheung, 1996; Park *et al.*, 1997; Kiho *et al.*, 2000)

2.1.1 Antitumor and Immunomodulatory Activity

Due to the dissatisfaction and occurrence of side effect with current cancer chemotherapy, polysaccharides extracted from mushrooms have become great interest as antitumor substances. As the fruiting body and liquid-cultured mycelium of higher Basidiomycetes have been found to contain polysaccharides that exhibit antitumor and immunomodulatory activities, therefore the discovery for new antitumor substances from mushrooms has become more and more popular.

In the last few decades, many of the mushroom polysaccharides extracted from higher Basidiomycetes have been subjected to various antitumor studies, for examples, polysaccharides derived from mushrooms *Lentinus edodes*, *Hericium erinaceum*, and *Ganoderma lucidum*.

Lentinan, which is derived from *Lentinus edodes*, is a purified (1→3)-β-D-glucan with (1→6)-β-D-glucopyranoside branches. It is successfully used as immunotherapeutical drug in clinical treatments for cancer patients. Lentinan administered at a dose of 1 and 5 mg/kg for 10 consecutive days after implantation of Sarcoma 180 cells in Swiss albino mice could significantly inhibited the growth of Sarcoma 180 solid tumor by 95.1 and 97.6%, respectively (Chihara *et al.*, 1970). Complete regression of Sarcoma 180 was also observed in 6 out of 10 mice treated with a dose of 1 mg/kg, and 7 out of 10 mice treated at a dose of 5 mg/kg. Other than in Swiss albino mice, lentinan could inhibit the growth of Sarcoma 180 by almost 100% at dose of 1 mg/kg in the mice model of SWM/Ms mice (Chihara *et al.*, 1970).

HE is a hot water extract isolated from *Hericium erinaceum*. Oral administered of HE could inhibit the Sarcoma 180 solid tumor in ICR mice at the dose of 500 mg/kg and 250 mg/kg, respectively, by 51.8% and 47.2%. However intraperitioneal administration of 100 mg/kg and 50 mg/kg of HE exhibited inhibition of Sarcoma 180 growth by 63.0% and 55.8%, respectively (Itoh *et al.*, 1999).

Moreover, mushroom polysaccharides GL-B, which was isolated from *Ganoderma lucidum* also showed significant antitumor effect in a dose dependent manner *in vivo* study. GL-B at dose of 50, 100 and 200 mg/kg, respectively, showed inhibitory activity on Sarcoma 180 cells in BALB/c mice by 27.70%, 55.83 & and

66.70% (Zhang & Lin, 1999).

Although many studies reveal mushroom polysaccharides could exhibit antitumor activity in animal model, it seems that the antitumor effect of mushroom polysaccharides is not due to the direct attack on tumor cells, but may produce their antitumor activities by activating different host immune responses.

According to the studies by Zhang & Lin (1999), GL-B could not inhibit the proliferation and induce apoptosis in HL-60 cells *in vitro* study. However in apoptosis analysis by using flow cytometry, apoptotic cells were observed in HL-60 cells, which have been incubated with the conditioned medium from GL-B activated macrophage (GL-B-M-CM) and the conditioned medium from GL-B activated T lymphocyte (GL-B-T-CM). This result illustrated that macrophage and T lymphocyte both contributed in inducing apoptosis by GL-B on HL-60 cells. Besides, mushroom polysaccharides MGLP1 and MGLP2, which were extracted from the mycelium of *Ganoderma lucidum*, also showed similar effect as that of GL-B (Hu & Lin, 1999). MGLP1 and MGLP2 both did not exhibit inhibitory effect on proliferation on HL-60 cells *in vitro*, while when MGLP1 and MGLP2 treated with the splenocyte in culture medium, it could induce HL-60 apoptosis. The result obtained from these two studies suggested that the antitumor effects of GL-B, MGLP1 and MGLP2 might be due to the activation of cytokine production by

macrophage, T-lymphocyte and splenocyte. As suggested by the author (Zhang & Lin, 1999), GL-B treated macrophage and T-lymphocyte might activate the production of TNF-α and IFN-γ. Whereas MGLP1 and MGLP2 treated splenocyte activated the production of IL-2, which could enhance the synthesis and secretion of INF-γ by T-lymphocyte. The INF-γ secreted may further activate the change of pre-cytotoxic T lymphocyte into mature cytotoxic T lymphocyte (Hu & Lin, 1999). TNF-α and IFN-γ have the ability in induction of apoptosis, whereas IFN-γ and cytotoxic T lymphocyte can inhibit the growth of tumor cells.

On the other hand, in the antitumor study of lentinan by using neonatal thymectomized mice, the inhibition on the Sarcoma 180 solid tumor was 6.4%, while the tumor inhibition rate in normal mice was 99.6%. This result also illustrated thymus has played an important role in the antitumor action of lentinan and it is responsible for the cell-mediated response in immune system that stimulated by lentinan as well (Maeda & Chihara, 1971).

2.1.2 Antiviral Activity

GLhw, which is a polysaccharides fraction isolated from the carpophores of *Ganoderma lucidum* by hot water extraction followed by ethanol precipitation, showed antiviral activity against herpes simplex virus type 1 (HSV-1) and herpes

simplex virus type 2 (HSV-2). GLhw showed cytopathic effect (CPE) inhibitory activity on confluent Vero cells with an EC_{50} of 1510 and 1790 μg/ml against HSV-1 and HSV-2, respectively, whereas the CC_{50} of GLhw on the host cells is 7860 μg/ml. Besides, GLhw inhibited plaque formations of HSV-2 on Vero and Hep-2 cells in a dose dependent manner with an EC_{50} of 590 and 580 μg/ml, whereas the CC_{50} of it are 880 and 1230 μg/ml respectively (Eo *et al.*, 1999).

Similar to GLhw, a polysaccharide protein complex isolated from the cultivated mycelium of *Coriolus versicolor* (PSK) also acts as an antiviral substance when administered intraperitoneally or intravenously into ICR mice. Pre-treatment or post-treatment of PSK is able to enhance the resistance to the infections of ectromelia virus and cytomegalovirus and prolong the survival time in mice model (Tsukagoshi *et al.*, 1984).

Pleuran, an alkali insoluble β-D-glucan isolated from *Pleurotus ostreatus*, has been reported to express antiviral activities against *Haemophilus influenze* as well. Intraperitional administration of pleuran promoted survival of mice susceptible to pulmonary *Haemophilus influenze* infection in a dose dependent manner (Karacsonyi & Kuniak, 1994).

Besides, lentinan derived form mushroom *Lentinus edodes*, when used in combination with azidothymidine (AZT), inhibit human immunodeficiency virus

(HIV) more effective than that of AZT alone (Tochikura et *al.*, 1987).

2.1.3 Hypoglycermic Activity

Hypoglycemic activity is also exhibited by mushroom polysaccharides Ganoderans A and B. These two polysaccharides are both obtained from hot water extraction followed by ethanol precipitation from the fruiting bodies of *Ganoderma lucidum.* Intraperitoneal injection of Ganoderan A and B in normal and alloxan-induced hyperglycemic mice elicited strong hypoglycemic and plasma sugar lever lowering activities (Hikino *et al.*, 1985)

2.1.4 Free Radical Scavenging Activity

Free radical scavenging activity is also observed from mushroom polysaccharides. The polysaccharide extracts from fruiting bodies of *Ganoderma lucidum* and *Grifola umbellata* inhibited the superoxide radical generation by 82.7% and 74.8% respectively in phenazin methosulphhate-NADH-nitroblue tetrazolium system at concentration of 80 μg/ml (Liu *et al.*, 1996a). Besides, the changes in proportion of protein in the polysaccharides extracts also affect the scavenging activity, as the polysaccharides to protein ratio decreased, the superoxide radical scavenging activity would be increased. PSK and polysaccharides extracts of *G.*

lucidum also inhibited the hydroxyl radical generation by 54.14% and 45.75% respectively in ascorbic acid-Cu^{2+}-cytochrome C system at concentration of 400 μg/ml (Liu *et al.*, 1996a).

2.2 Mushroom *Dictyophora indusiata* (竹蓀)

Dictyophora indusiata is referred as a "long veiled lady mushroom", because its appearance looks like a lady wearing a long dress. It is a very beautiful, phallic shaped mushroom, which is widely distributed in China (known as Zhu Sun), Japan (known as Kinugasatake), Africa as well as South America. *D. indusiata* is an edible mushroom belongings to the class Basidiomycetes, order Phallales and family Phallaceae (徐錦堂, 1997). It has been reported to have antihypertensive, blood lipid lowering, cholesterol lowering (徐錦堂, 1997; 張樹庭及卯曉嵐, 1995) and antimicrobial activities (Tan & Hu, 1999). In addition, *D. indusiata* is also used as folk remedies for the treatment of dysentery (徐錦堂, 1997).

Since *D. indusiata* has been used as a nutritious food and medicinal drug in China, there are various studies in examining the nutritional value, structural characteristic and biological activities of this mushroom and its isolated polysaccharides

2.2.1 Nutritional Value

Dictyophora indusiata have been considered as a nutritious food and one of most delicious mushrooms. According to a study, twelve inorganic elements, vitamin E, fifteen amino acids (including valine, leucine, threonine, methionine, lysine and

pheuylalanine were determined in this mushroom (Ouyang *et al.*, 1998).

2.2.2 Structural Characteristic of *Dictyophora indusiata* Polysaccharides

Six homogenous polysaccharides (T-2-HN, T-3-M, T-3-G, T-4-N, T-5-N, and T-3-Ad) and a conjugated polysaccharide fraction (T-2-A) have been obtained from the fruit bodies of mushroom *D. indusiata* in various studies.

T-2-HN and T-3-M both were partially *O*-acetylated (1→3)-α-D-mannan. T-2-HN and T-3-M were protein-free polysaccharides, since they were deproteinized in the isolation process by the Pronase treatment and Sevag method (Ukai *et al.*, 1980; Hara *et al.*, 1986). According to the methylation analysis by Hakomori procedure, T-2-HN was a linear chain composed of α-1→3 linked D-mannopyranosyl residues (>97%) and traces of 1→6 linked D-mannopyranosyl residues (Ukai *et al.*, 1980), whereas T-3-M composed of 2,4,6-tri-*O*-methyl-D-mannopyranose with a negligible proportion of 2,3,4,6-tetra-*O*-methyl-D-mannose (Hara *et al.*, 1988). On the other hand, the result obtained from periodate oxidation and Smith degradation showed that T-2-HN contains appoximately one acetyl group (11.4%) per two D-mannosyl residues in the α-(1→3)-linked D-mannopyranosyl chains and 88% of the acetyl groups were located on O-6 of the D-mannosyl residues (Hara *et al.*,1982). Besides, T-3-M

contained α- (1→3)-linked D-mannopyranosyl residues (Hara *et al.*, 1988). In addition, the molecular weight of T-2-HN and T-3-M were estimated by gel chromatography on a Sepharose 2B column and Sepharose CL-2B respectively. The molecular weight of T-2-HN (~6.2×10^5) is higher than that of T-3-M (~1.9×10^5).

Apart from the partially *O*-acetylated (1→3)-α-D-mannan, β-D-glucans (T-3-G, T-4-N and T-5-N) were also isolated from *D. indusiata*. T-3-G, T-4-N and T-5-N were isolated from a hot-water extract (Hara *et al.*, 1986), 2% sodium carbonate extract (Hara *et al.*, 1983), and alkaline (1N sodium hydroxide) extract (Ukai *et al.*, 1982) of the fruit bodies of *D. indusiata* respectively. Since these three polysaccharides were β-D-glucans, they all possessed a main chain of β-(1→3)-linked D-glucopyranosyl residues with branches consisting of a single β-(1→6)-linked D-glucopyranosyl groups attached to the main chain. However, the molecular weight and location of the side chain were different in these three β-D-glucans. The molecular weight determined by gel filtration on Sepharose CL-2B using 0.25 M sodium hydroxide as eluant were 5.1×10^5, 5.5×10^6 and 3.3×10^5 in T-3-G, T-4-N and T-5-N respectively. Besides, the side chains β-(1→6)-linked D-glucopyranosyl groups of T-3-G and T-4-N were attached on average to every fifth sugar residue, whereas that of T-5-N were attached on average to every seventh sugar residue of the main chain (Ukai *et al.*, 1982; Hara *et al.*, 1983; Hara *et al.*,

1986). Moreover, as reported by Hara *et al.* (1986), T-3-G might be one of the cell-surface glycans of *D. indusiata*, and T-4-N and T-5-N might be the components that were present either in the cell wall or inside the cells of *D. indusiata* (Hara *et al.*, 1986).

Other than T-3-G, a conjugated polysaccharide fraction, fucomannogalactan (T-3-Ad) was also isolated from the hot-water extract of fruit bodies of *D. indusiata*. T-3-Ad is a heterogalactan, which was composed of L-fucose, D-mannose and D-galactose (Hara *et al.*, 1991).

2.2.3 Biological Activity

Dictyophora indusiata polysaccharides have been reported to possess anti-inflammatory (Hara *et al.*, 1982b), mitogenic, colony stimulating factor-inducing (Hara *et al.*, 1991) and antitumor activities (Ukai *et al.*, 1983).

T-5-N was able to inhibit carrageenan-induced edema and scalded edematous hyperalgesia in Sprague Dawley (SD) rat's hindpaw at dose of 12 or 25 mg/kg (i.p. × 2) and 25 mg/kg (i.p. × 2) respectively. The result showed the effects of T-5-N were stronger than 25-50 mg/kg (i.p. × 2) of phenylbutazone (Hara *et al.*, 1982b).

Table 2.1 *Dictyophora indusiata* polysaccharides.

Polysaccharides	Polysaccharides Type	Molecular weight (Dalton)	Reference
T-2-HN	O-acetylated (1→3)-α-D-mannan	6.2×10^5	Hara *et al.*, 1982a Ukai *et al.*, 1980
T-3-M	α-(1→3)-linked-D-mannan	1.9×10^5	Hara *et al.*, 1988
T-3-G	(1→6)-branched (1→3)-β-D-Glucan	5.1×10^5	Hara *et al.*, 1986
T-4-N	(1→6)-branched (1→3)-β-D-Glucan	5.5×10^6	Hara *et al.*, 1983
T-5-N	(1→6)-branched (1→3)-β-D-Glucan	3.3×10^6	Ukai *et al.*, 1982
T-3-Ad	Fucomannogalactan	1.4×10^4	Hara *et al.*, 1991
T-2-A	Conjugated polysaccharide fraction	---	Hara *et al.*, 1991

Moreover, it was proved that the polysaccharide fractions extracted from the fruit bodies of *D. indusiata* were able to enhance the immune response. T-3-Ad, T-4-N and T-2-A could exhibit mitogenicity for murine lymphocytes and colony stimulating factor (CSF)-inducing activities in blood of mice. Among these three polysaccharides extracts, T-2-A was showed to have the most potent effect (Hara *et al.*, 1991).

In additional to the mitogenic and CSF-inducing activities, *D. indusiata* is known to contain polysaccharide compounds having antitumor activity when intraperitoneally administered. T-2-HN, T-4-N and T-5-N were shown to exhibit anti-tumor activity against Sarcoma 180 ascites cells. In the experiment done by Ukai *et al.*, (1983) intraperitoneal administration of T-2-HN at dose of 25 mg/kg/d ×10, T-4-N and T-5-N at dose of 10 mg/kg/d ×10 gave inhibition rates of 77%, 25% and 77% of the control respectively (Ukai *et al.*, 1983).

2.3 *In vivo* Antitumor Study

The use of Sarcoma 180 cells has been well established for *in vivo* antitumor studies, though the animal model, site and route of tumor cells implantation and the period of treatment are different in many studies (Liu *et al.*, 1995; Mizuno *et al.*, 1995; Itoh *et al.*, 1999; Ohno *et al.*, 2000). Although most of the studies are anti-solid S-180 tests, the anti-ascitic S-180 activity of the culture filtrates from *Schizophyllan commune* and *Tricholoma* sp. was also evaluated in additional to anti-solid S-180 activity (Komatsu *et al.*, 1969; Liu *et al.*, 1995).

The researches always use mice as their animal model, since they are easy to handle and have a rapid breeding cycle. The commonly used mouse models are BALB/c and ICR mice. The best sites for tumor cells implantation are intramuscular (i.m.) in the leg or subcutaneous (s.c.) over the lower back of the mice, since the tumor size can be measured easily (Begg, 1987). Besides, the period of treatment and the whole experiment are different in many studies; it usually ranged form 10 days to 18 days and 14 days to 35 days respectively. For example, HE, a hot-water extract of *Hericium erinaceum* was administered to ICR mice starting on the 7^{th} day before the implantation of tumor cells once daily for 18 days, and the tumor was excised 14 days after the implantation. On the other hand, the 1,3-β-glucan isolated from cultured fruit body of *Sparassis crispa* was administered for 10 consecutive

days from 24 hours after the tumor inoculation, and the tumor was excised 35 days after tumor inoculation. Besides, the anti-tumor effect has been evaluated by using different parameters, such as percentage of tumor inhibition, regression of tumor and mortality of the tumor bearing mice.

2.4 Induction of Cytokines Production in Immune System

Cytokines are a diverse group of small soluble glycoprotein (<30kDa) (Wise & Carter, 2002), and its main function is to act as intercellular signals within immune system (Kelso, 1998). They encompass families of regulators, including interleukins (IL), tumor necrosis factors (TNF), interferons (IFN) and certain growth factors, and are produced by every nucleated cell in the body. Besides, Cytokine synthesis is a consequence of cellular activation, which is induced by a various kinds of exogenous and endogenous stimuli, depending on the nature and the differentiation or activation state of the secreting cell; for example, antigen or antigen-MHC can trigger the cytokines production by B or T cell (Kelso, 1998).

Since most of the antitumor effects *in vivo* are due to immunomodulatory activity rather than direct cytotoxicity, the measurement of cytokines production is applied in order to study the regulation of cytokines in the host immune system. Cytokine expression, synthesis and secretion were investigated by various techniques in different researches, including: cytokine-specific mRNA expression analysis and the assay of secreted cytokines in supernatant or biological fluid.

In the analysis of cytokine-specific mRNA expression, both Northern blotting as well as reverse transcriptase polymerase chain reaction (RT-PCR) were applied in different studies. Northern blot analysis was used by Liu *et al.* (1993) to study the

expression of TNF by adherent peritoneal macrophages collected from polysaccharopeptide (PSP) treated C57 mice (Liu *et al.*, 1993). Besides, by using RT-PCR techniques, the cytokine mRNA can be isolated with the aid of specific primer. For example, the immunomodulating activity of mushroom polysaccharides was evaluated by using RT-PCR. The cytokine gene expression of polysaccharide-protein complex (PSPC) and lentinan was compared by RT-PCR using primer specific for IL-1α, IL-1β, TNF-α, IFN-γ and macrophage-colony stimulating factor (M-CSF) (Liu *et al.*, 1999). According to the result, both of the mushroom polysaccharides were able to up-regulate IL-1α, IL-1β, TNF-α, IFN-γ and M-CSF in ICR mice. Moreover, the cytokine gene expression induced by a highly branched (1→3)-β-D-glucan (OL-2) administration in different mice model (ICR and AKR mice) were also compared by RT-PCR by Nemoto *et al.* (1994). Since AKR mice is a strain with a genetic C5 deficiency, the difference pattern of cytokine gene expression induced by OL-2 in ICR and AKR demonstrated the complement cascade was contributed in OL-2- induced cytokine gene expression (Nemoto *et al.*, 1994).

Different cytokines may be present in biological fluid (including serum, plasma) or culture supernatant, therefore the assay of secreted cytokines is used by researchers as well. The determination of secreted cytokines, which is based on the

bioactivity and receptor-binding properties of the cytokines, were carried out by using indicator cells and enzyme linked immunosorbent assay (ELISA), respectively.

As each different cytokine may have its specific biological activity, a suitable indicator has to be employed in order to identify a particular cytokine. The proliferative response of the indicator cells is usually assessed by [^{3}H] thymidine uptake, MTT reduction assay or crystal violet staining. For example, in the study of mushroom lectins from *Tricholoma mongolicum* on immunomodulatory activity (Wang *et al.*, 1996b), established cell line L929 mouse fibroblast has been used for measuring the biological activity of TNF. The proliferation response of L929 was defined as percent inhibition of L929 growth by crystal violet staining. And in the *in vitro* study of a purified (1→3)-β-D-glucan (GRN) in activation of cytokine production, which was isolated from the mushroom *Grifola frondosa*, the production of IL-1 in the culture supernatants and the macrophage cell lysates was determined in terms of the thymocyte proliferation response by [^{3}H] thymidine uptake by Adachi *et al.* (1994). The indicator cells thymocytes used in this experiment were freshly isolated from C3H/HeJ mice (Adachi *et al.*, 1994), whereas L929 is an established cell line.

Due to the availability of large amounts of recombinant cytokines and monoclonal anti-cytokine antibodies, sandwich type of ELISA has been applied in

the immunoassay of cytokines. In this immunoassay, the measurement of cytokine is based on the colorimetric determination of enzyme activity (for example, avidin-alkaline phasphatase or avidin-horseradish peroxidase) coupled to the detecting antibody. In a recent study by Tokunaka *et al.* (2000), ELISA was used to study the immunopharmacological activity of a *Candida* water-soluble (1→3)-β-D-glucan (CSBG). In this study, rat monoclonal anti-mouse TNF-α and rabbit polyclonal anti-mouse TNF-α antibodies were used for capture and detection of TNF-α in mouse serum, respectively. CSBG is able to prime the production of TNF-α after lipopolysaccharides (LPS) administration (Tokunaka et al., 2000) as most β-glucan (GRN, SPG, SSG and ZYM) in the study by Ohno *et al.* (1995).

2.5 *In vitro* Antitumor Study

In antitumor study *in vitro*, different means for quantifying cell population can be utilized to assess the proliferation and cytotoxicity of the drugs on cancer and normal cell lines respectively, for example, direct cell count, colorimetric measurement and [^{3}H]-thymidine/ BrdU incorportion.

Cell density and viability can be determined by the actual enumeration of cells by direct microscopy or automated cell counting. Trypan blue exclusion method is the most primitive, simple and widely used method in direct cell count. This was performed by counting cells of preparations with a microscopic counting chamber (hemocytometer) in the presence of trypan blue stain. Since trypan blue is a vital dye and can leak into cells that have damaged cell membrane, viable cells exclude the dye while dead cells are stained dark blue in color (McAteer & Davis, 1994). Besides, Coulter counter is also a widely used device for counting cells. In this method, cells drawn through a fine orifice increase the electrical impedance to the current flowing and produce a series of pulses that are sorted and counted (McAteer & Davis, 1994).

Other than direct cell count, colorimetric method including MTT assay and measurement of LDH activity was also applied for cell quantification and cytotoxicity respectively. In 1983, Mosmann described a rapid colorimetric assay for

the measurements of cell growth. The assay is based on the cleavage of the yellow water-soluble substrate 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a water insoluble dark blue formazan product by mitochondrial dehydrogenase enzymes in living cells (Mosmann, 1983). The amount of formazan produced is directly proportional to the cell number (Gerlier & Thomasset, 1986). In addition, the cytotoxity can be assessed by LDH activity as well. This assay is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. The LDH activity can be measured colorimetrically in an enzymatic test.

Apart from colorimetric method, [^{3}H]-thymidine incorporation is one of the most traditional methods for quantifying cell proliferation. Since chromosome replication is accompanied by the incorporation of soluble nucleotide precursor into newly synthesized DNA in the S phase in cell cycle, the monitoring of DNA synthesis is another indirect parameter of cell proliferation. In this method, dividing cells are pulsed with radioactive exogenous precursor [^{3}H]-thymidine, the amount of radioactivity incorporated into the cell DNA is determined by harvesting the cells on a glass-fiber filter followed by liquid scintillation counting. Due to the potential hazards associated with the use of radioactive substances, non-radioactive method alternative to the [^{3}H]-thymidine incorportion was developed (Takeuoshi *et al.*,

2001). [^{3}H]-thymidine is replaced by 5-bromo-2'-deoxyurdine (BrdU) in this method and it is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells.

2.6 Cell Cycle Regulation

In eukaryotic cells, the cell cycle consists of a series of stages, termed G_1, S, G_2 and M, in which cells have to pass through consecutively. The principal task of the cell cycle is to duplicate chromosomal DNA precisely during S phase and to segregate it into two identical progeny during M phase (mitosis). Besides, G_1 and G_2 phases are between S and M phases. G_1 phase is a period distinguished by cell growth, and G_2 phase has been described as an interval in which the cells prepare for division (Becker *et al.*, 2000; Sherr *et al.*, 2000).

Located between G_1 and S, G_2 and M phases are two checkpoints, G_1 (restriction point) and G_2 checkpoint. These checkpoints are the preparatory periods during which the cell is growing and/or synthesizing the factor required during S and M phases respectively. They are responsible for checking the specific activities, such as the growth in cell size or DNA replication, which have to be carried out properly during the phase before the checkpoint. If there are any improper activities, the cell cycle will stop at these checkpoints temporarily and amends any possible errors, or the cell will withdraw from the cell cycle into a quiescent state, G_0 phase (Becker *et al.*, 2000).

Generally, the cell cycle control is provided by a family of serine/threonine protein kinase complexes consisting of a catalytic subunit, a cyclin dependent kinase

(cdk); and a regulatory subunit, cyclin (Norbury & Nurse, 1992). And the cyclin-cdk activity is regulated by the availability of cyclin subunit, state of phosphorylation of cyclin-cdk complex and two groups of cdk inhibitors (INK4 and Cip/Kip families) (Molinari, 2000).

Cdks subunits by themselves are inactive, and binding of cdk subunit to a cyclin protein is required for their activity as well as their regulation. These complexes undergo changes in the kinase and cyclin components that are believed to drive the cell from one stage of the cell cycle to another. D type cyclins with cdk4 or cdk6, cyclin E with cdk2, cyclin A with cdk2 and cyclin B with cdk1 are responsible for the progression through G1, G1/S, S and G_2/M phase of the cell cycle respectively (Zetterberg, 1996).

Apart from the cyclin, cyclin dependent kinase and cyclin dependent kinase inhibitor, tumor suppressor proteins are also involved in cell cycle regulation, for examples, the Rb and p53 proteins.

Rb protein with a molecular weight of 105 kDa is encoded by Rb gene that is the first tumor suppressor gene to be identified in hereditary retinoblastoma (RB). Besides, Rb is a component of G1 signaling pathway and the role of Rb is to control the transition from G1 into S phase (Weingberg, 1995). In its hypophosphorylated state, Rb prevents progression from G1 to S phase by binding with members of the

E2F transcription factor family. The binding of Rb with the transcription factor E2Fs (including E2F1, 2 and 3) not only blocks transcription activation by E2F but also actively represses transcription by recruiting histone deacetylase to the promoter of genes required for S phase entry (Brehm *et al.*, 1998).

On the other hand, p53, which is also a tumor suppressor protein, regulate the cell cycle by determining either cell cycle to arrest, allowing the repair of damaged DNA, or to undergo programmed cell death (apoptosis). When the genome suffers damage that cannot be repaired, p53 would mediate an apoptotic response that eliminates the threat of the deregulated cell. But if the damage is not severe, p53 can induce production of a cyclin dependent kinase inhibitor p21, thereby obstructing cyclin D- and cyclin E-cdk activity, and halting the cells at the G1 restriction point for DNA repair (Becker *et al.*, 2000).

Defects in the cell cycle regulatory mechanism such as uncontrolled cell proliferation, decreased cellular differentiation, ability to invade surrounding tissue, and ability to establish new growth at ectopic sites (metastasis) are the properties of cancer cells that are different from normal cells. For example, leukemia arises as uncontrolled proliferation that occurs in hematopoietic cells. As such, it seems that the effect of some antitumor agents may contribute by restoration of cell cycle regulation by cell cycle arrestment and induction of apoptosis on defected cells.

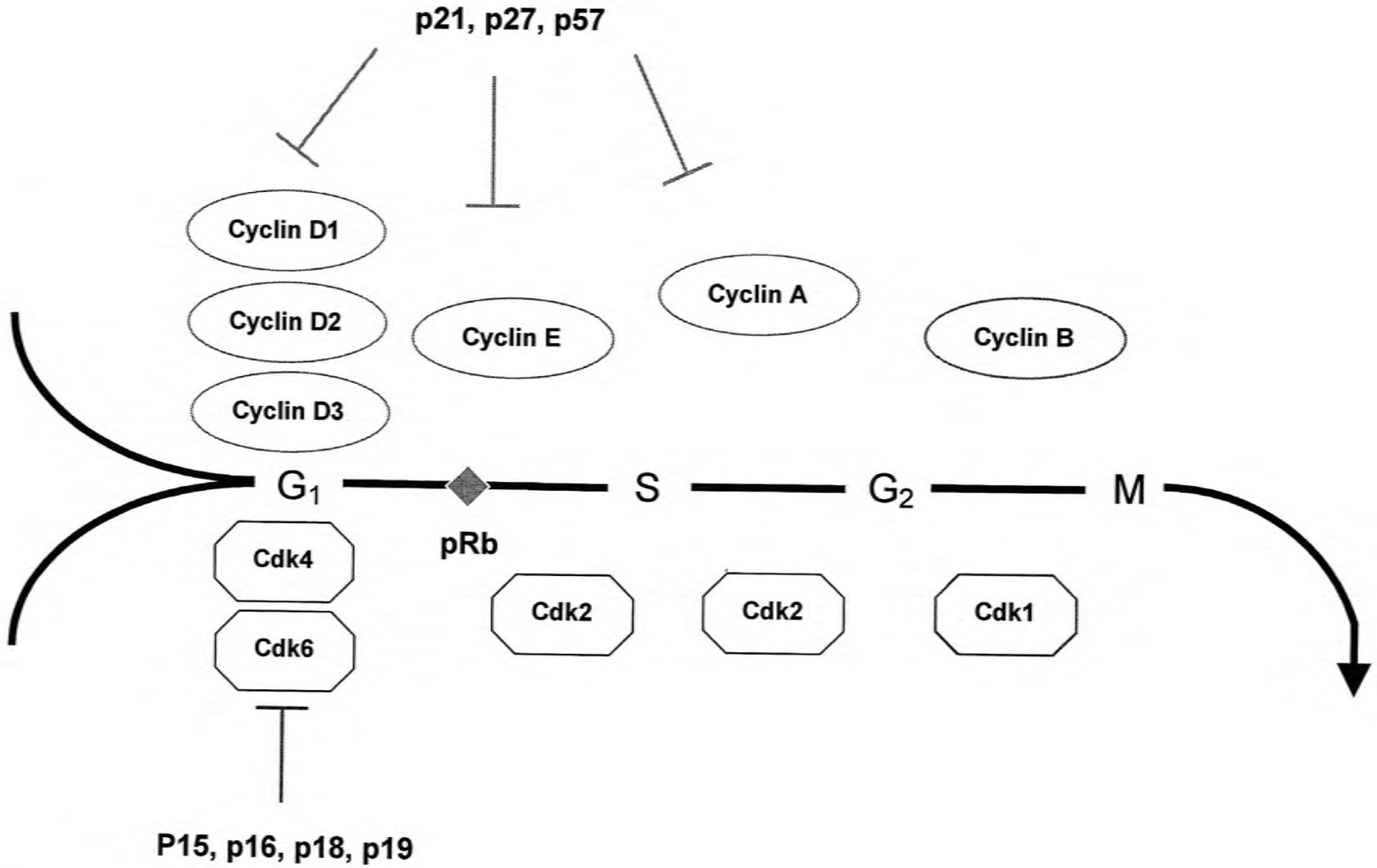

Fig. 2.1 Regulation of the cell cycle

Progression through the cell cycle is positively regulated by the cyclin-Cdks complex. The activities of the kinase are negatively regulated by two groups of cyclin dependent kinase inhibitors. (Modified from Hall & Peter, 1996)

In a recent study, Connor *et al.* (2001) demonstrated the G_1 cell cycle arrest after the treatment of ceramide in the leukemic cell lines. The result obtained from Western blotting analysis revealed that Rb might play a key role as the effector of ceramide signaling, since Rb activation (appearance of hypophosphorylated forms of Rb) was observed in the ceramide responsive cells. Therefore, it illustrated that ceramide is a key regulator of cellular proliferation by preventing progression of leukemia cell from G_1 to S phase (Connor *et al.*, 2001).

Besides, a natural product Bu-Zhong-Yi-Qi-Tang (BZYQT), which was extracted from a formulation of ten Chinese medicinal herbs, inhibited proliferation of three of the human hepatoma cell lines, including Hep3B, HepG2 and HA22T. The IC_{50} of BZYQT on Hep3B, HepG2 and HA22T are 432.5, 455.4 and 2284.3 μg/ml respectively. Besides, in the analysis of cell cycle by using flow cytometry and gel electrophoresis of fragmentation DNA, apoptotic (sub-G_1) peak in the DNA histogram and DNA fragmentation in a ladder pattern were observed in Hep3B cell lines. The result illustrated the ability of BZYQT in inhibiting cell cycle progression in hepatoma cells followed by apoptosis (Kao *et al.*, 2001).

In addition to BZYQT, magnolol, which is a hydroxylated biphenyl compound, suppressed human colon (COLO-205) and liver cancer (Hep-G2) cells in *vitro* as well. Magnolol was extracted from a widely used Chinese herbal medicine *Magnolia*

officinalis. Similar as that of BZYQT, magnolol treated COLO-205 and Hep-G2 cells displayed the DNA ladder patterns in the DNA gel electrophoresis. Moreover, magnolol inhibited [^{3}H] thymidine incorporation during the S phase of the cell cycle as well (Lin *et al.*, 2002).

Although the mechanism has not been well studied, higher Basidiomycetes mushrooms were shown to have anti-proliferative activity on leukemia cells *in vitro*. The suppressive effect of *Ganoderma lucidum* Karst compound (GLC) on K-562 proliferation is not only in a dose-dependent (from 4 to 20 mg/ml) but also in a time-dependent manner (from 1 to 5 days) (Zhong *et al.*, 1999).

Chapter 3

Materials and Methods

3.1 Extraction

3.1.1 Extraction of *Dictyophora indusiata* Polysaccharides

Dried fruit bodies of *Dictyophora indusiata* were purchased from local market (Fig. 3.1). After being soaked and washed with 2 liters of distilled water, the fruit bodies (200 g) were disintegrated with a blender and extracted with hot water in a slow cooker for 2-3 hours. The water extracts were percolated with gauze and the residues were boiled again with 2 more liters of distilled water. This hot water extraction was repeated for 2 more times, and each time last for 2 to 3 h as before. The water extracts obtained were pooled and centrifuged at 22,095 g for 30 min with a J2-MI centrifuge (Beckman, USA) and a JA-14 rotor to remove water-insoluble components. The collected supernatant of the extracts was concentrated by rotary evaporator (BÜCHI Rotavapor R-200, Switzerland) and precipitated by 40% ethanol

to obtain the relatively higher molecular weight polysaccharides. The relatively lower molecular weight polysaccharides were obtained by 80% ethanol precipitation. To allow more times for complete precipitation, the extracts were kept overnight at 4°C. The precipitates formed from these two ethanol precipitations were collected by centrifugation at 22,095 g for 30 min. The pellets were re-dissolved in distilled water and lyophilized. The two lyophilized products obtained from 40% and 80% ethanol precipitations were designated as DI2 and DI3 fractions, respectively.

3.1.2 Purification of *Dictyophora indusiata* Polysaccharides

3.1.2.1 Preparation of DEAE-cellulose Ion Exchanger

DEAE-cellulose resin (Sigma, USA) was suspended in distilled water and left to swell overnight at 4°C. The resin was first treated in 2 volumes of 0.4 N HCl solution for 30 min. After discharging the HCl solution, the resin was washed with distilled water until pH was close to 7.0. The resin was further treated in 2 volumes of 0.4 N NaOH for 30 min. After discharging the NaOH solution, the resin was also washed with distilled water until the pH was near to 7.0.

3.1.2.2 Fractionation

Fifty miligrams of crude DI3 polysaccharides dissolved in distilled water was

applied onto a DEAE anion exchange cellulose column [2.5 cm (I.D.) × 30 cm (L)]. The column was first washed with distilled water to elute the unbound polysaccharides, which were designated as DI3a fraction. The retained components were then eluted with a linear gradient of sodium chloride solution ranging from 0 to 1.0 M. Fractions of 5 ml/tube were collected at a flow rate of 20 ml/hr. Presence of polysaccharides in each tube was determined by phenol-sulfuric assay (Dubois *et al.*, 1956). The concentration of sodium chloride solution, which was used to elute the retained polysaccharides, was calculated by the following equation:

$$C = 1 + 1 \times \frac{\text{Volume eluted by particular concentration of NaCl solution}}{\text{Total volume eluted}}$$

The retained components, which were eluted by the concentrations of 0.04 M and 0.5 M NaCl solutions, were designated as DI3b and DI3c fractions, respectively. Desalting of DI3 fractions was performed by dialysis (Spectra/Por®, MWCO: 12-14,000) against distilled water at 4°C. The remaining solution was then lyophilized. A simplified flow chart illustrating the procedures of extraction and purification of the polysaccharides is shown in Fig. 3.2.

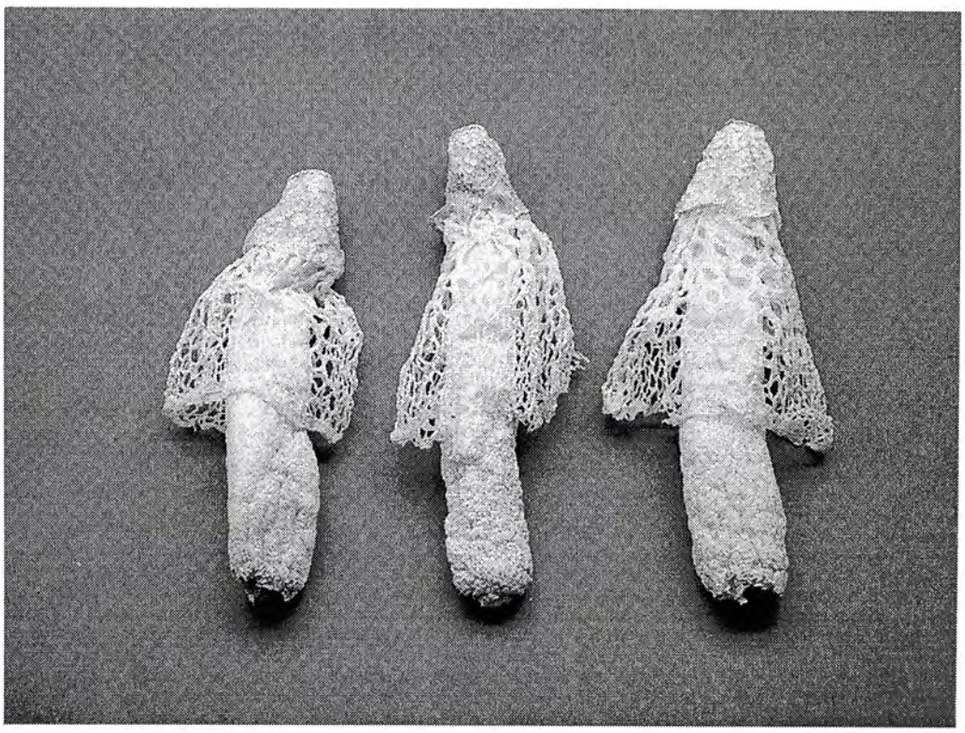

Fig. 3.1 Dried fruit bodies of *Dicytophora indusiata* purchased from local market.

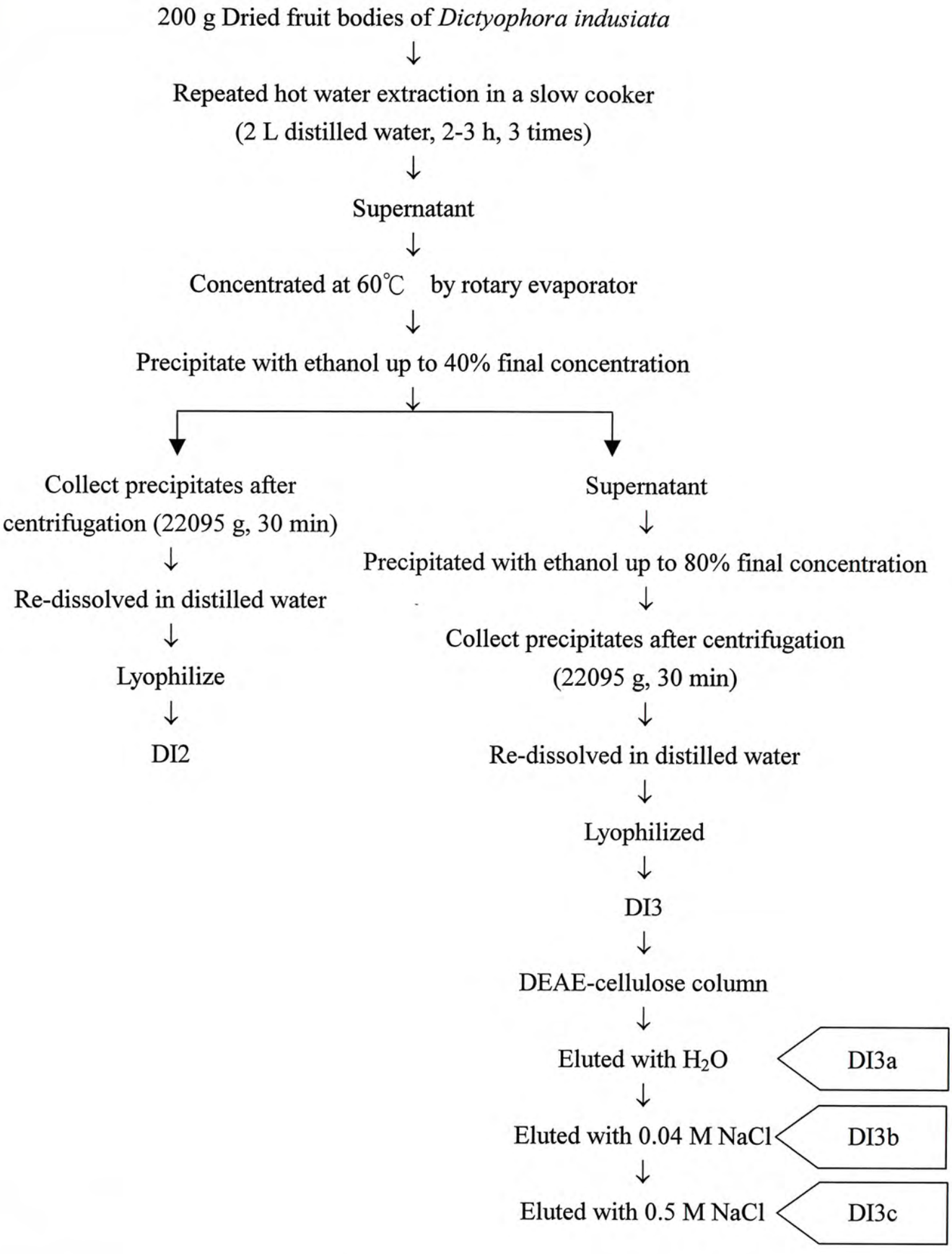

Fig. 3.2 A simplified flow chart showing the extraction and purification of *Dictyophora indusiata* polysaccharides.

3.2 Characterization of *Dicytophora indusiata* Polysaccharides

3.2.1 Polysaccharide Content Determination

Contents of polysaccharides in DI and DI3 fractions were determined by Phenol sulfuric assay (Dubois *et al.*, 1956; Scherz & Bonn, 1998). Reactions of polysaccharides with phenols, in the presence of sulfuric acid, are universal, and are suitable for both neutral and acidic polysaccharides.

In this study, 0.2 ml of 5% phenol solution was added to 0.2 ml of 100 μg/ml aqueous sample (both of them were dissolved in distilled water). A blank control, which consisted of distilled water only, and a standard curve of D-glucose (Ajax) ranging from 0-70 μg/ml, were also prepared. 1 ml of concentrated sulfuric acid (98.8 %) was added carefully to the surface of the solution, and the mixture was allowed to stand for 10 min at room temperature. After intensive vortexing, the mixture was allowed to stand for another 30 min at room temperature. The resultant solution was orange-red in color, and its absorbance, against the blank control, was measured at wavelength 490nm with a microplate reader (SPECTRAmax 250).

3.2.2 Protein Content Determination

Content of proteins in DI3 fraction was determined by Lowry-Folin method (Lowry *et al.*, 1951; Waterborg & Matthews, 1994), which was based on both the

Biuret and Folin-Ciocalteau reaction.

In this study, 0.1 ml 2 N NaOH solution was added to 0.1 ml of 1 mg/ml aqueous sample (both of them were dissolved in distilled water). A blank control, which consisted of distilled water only, and a standard curve of bovine serum albumin (Sigma) ranging from 0-500 μg/ml were also prepared. The mixture was hydrolyzed at 100°C for 10 min in a heating block. After cooling to room temperature, 1 ml of freshly mixed complex-forming reagent, which was prepared by mixing 2% Na_2CO_3, 1% $CuSO_4$ $5H_2O$ and 2% sodium potassium tartrate (w/v) in distilled water in a volume ratio of 100:1:1, was added to the mixture. After standing for 10 min at room temperature, 0.1 ml of Folin-Ciocalteu's phenol reagent (BDH) was added to the solution. The mixture was then allowed to stand for 30 min at room temperature. The absorbance of the solution was then measured at wavelength 750 nm with the microplate reader.

3.2.3 Gas Chromatography (GC)

DI3 fraction was subjected to Gas Chromatography (GC) to determine the composition of neutral monosaccharides. About 13 mg of the sample was hydrolyzed with 0.7 ml 12 M sulfuric acid at 35℃ for 1 h with constant stirring. The hydrolysate was then diluted to 2 M sulfuric acid with 3.5 ml distilled water, and

boiled in a water bath with continuous shaking for 1 h. After cooling to room temperature, 3 ml of the hydrolysate was used to prepare alditol acetates of neutral and amino sugars according to the method described by Blankeney *et al.* (1983). The remaining hydrolysate was reserved for uronic acid determination in Section 3.2.4. β-D-allose (1 mg/ml) was added into the hydrolysate as an internal standard. 1.25 ml ammonia (12 M) and 5 µl octan-2-ol was added to neutralize the hydrolysate and prevent foam forming, respectively. The hydrolysate was then reduced by adding 0.2 ml freshly prepared sodium borontetrahydride (200 mg/ml) and incubating in 40℃ water bath for 30 min. 0.1 ml glacial acetic acid was added to stop the reducing reaction, which was followed by 2 ml acetic acid anhydride and 0.3 ml 1-methylimidazole. The mixture was vortexed and left at room temperature for 10 min. 5 ml distilled water was then added to dissolve the excess acetic anhydride. After adding 1 ml dichloromethane, the mixture was allowed to stand for 10 min for phase separation. While the top aqueous layer was removed, the bottom organic layer was washed twice each with 2 ml distilled water. Anhydrous sodium sulphate was then added to dehydrate the layer. The obtained sample solution was then stored in a vial at -20°C prior to use. Determination of neutral sugar with GC was performed with HP6890 gas chromatography using an Alltech DB-225 capillary column [15 m (L) × 0.25 mm (I.D.), 0.25 µm film thickness]. The oven temperature

program was given as follows: initial temperature, 180°C with 4°C /min rise to 220°C and held at 220°C for 30 min. Helium was used as the carrier gas in the set up. 2 µl sample was injected into the column and the neutral sugar in the sample was detected by flame ionization. The amount of monosaccharides present in each sample was expressed as proportion by weight (%w/w).

3.2.4 Uronic Acid Content Determination

The content of uronic acid was determined by colorimetric method according to the Officical Methods of Analysis (45.4.11) (AOAC, 1996). Hydrolysate obtained from acid depolymerization was used for determination. 300 µl of boric acid-sodium chloride solution (2% sodium chloride and 3% boric acid in distilled water) was added to an aliquot of 300 µl hydrolysate in a test tube and mixed thoroughly. A blank control consisting of sulfuric acid (2 M) and D-galacturonic acid monohydrate standards (10, 50, 100, 200, 500 µg/ml) were prepared, respectively. 5 ml concentrated sulfuric acid (12 M) was added to the mixture with vortexing, which was then kept at 70°C for 40 min. After the mixture was cooled to room temperature, 200 µl of dimethyphenol (0.1% 3,5-dimethyphenol in glacial acetic acid) was added and vortexed thoroughly for 5 min. The mixture was then stood at room temperature for 15 min. Absorbance of the samples solutions were measured against blank at 400

and 450 nm, respectively. Readings at 400 nm were subtracted from those at 450 nm to correct any interference from hexoses. The uronic acid content was determined using D-galacturonic acid monohydrate as standard, and expressed as a polysaccharides residue by multiplying a factor of 0.83. The uronic acid content present in each sample was expressed as proportion by weight (%w/w).

3.2.5 High Performance Liquid Chromatography (HPLC)

Purity and molecular weight of DI3 fraction were determined by analytical High Performance Liquid Chromatography (HPLC). Samples (10 μg) were dissolved in 0.5 M NaCl solution and filtered through a 0.22 μm filter. HPLC was carried out using Hewlett Packard series 1100 HPLC system equipped with a TSK gel^{TM} column G5000PW [30 cm (L) × 7.5 mm (I.D.), particle size: 17 μm] (SUPELCO). DI3 fraction was eluted using 0.5 M NaCl at a flow rate of 0.5 ml/min at 25°C, and detected with Refractive Index detector (HP 1047A RI Detector). Dextran standards of molecular weight 1, 5, 25, 80, 270 and 670 kDa, respectively, were used for estimating the molecular weight of polysaccharides in the sample.

3.3 *In vivo* Studies

3.3.1 Animals

Male BALB/c mice (8-9 weeks) were used in this study. All the mice were supplied by the Laboratory Animal Service Centre (LASEC) of The Chinese University of Hong Kong. The animals were housed in the Animal House of Department of Biology (CUHK) under the normal laboratory conditions (21 ± 2°C, 12/12 h light/dark cycle). They were freely accessible to tap water, and maintained on standard rodent chow. All the mice were allowed to acclimatize for a week before each experiment.

3.3.2 Maintenance of Sarcoma 180 Cell Line

Sarcoma-180 cell line in ascitic form was maintained in male BALB/c mouse peritoneum, and was passed to another mouse at every seventh day. After the mouse was killed by cervical dislocation, the Sarcoma 180 cells in ascitic fluid were collected by lavage of peritoneal cavity with sterile PBS. The cells were then washed twice and resuspended in sterile PBS. The viable cells were counted by hemocytometer and Trypan blue exclusion method. After adjusting the cell density to 2.5×10^7 cells/ml by sterile PBS, 0.2 ml of Sarcoma 180 cells suspension (i.e. 5×10^6 cells/0.2 ml) was passed to another mouse (i.p.).

3.3.3 Effect of DI3 Fraction on Sarcoma 180 Solid Tumor (Ohno *et al.*, 1984)

The antitumor effect of DI3 fraction was studied *in vivo* using male BALB/c mice (~20-25 g). At the beginning of the experiment, a suspension of Sarcoma-180 cells was prepared as described in Section 3.3.2 After anesthetization of the mice, initial body weight of each mouse was recorded, and 0.2 ml of Sarcoma-180 cells were inoculated subcutaneously onto the back of each mouse. Three days after inoculation, the tumor-bearing mice were separated into groups and caged randomly. DI3 fraction was dissolved in PBS at different doses, and was administered to the tumor-bearing mice for 10 consecutive days (i.p.). Control group with tumor-bearing mice was given sterile PBS only. Another control group, which was neither implanted with tumor cells nor received peritoneal injection of samples, was also setup to monitor the weight change of normal mice. Twenty-one days after inoculation, the mice were sacrificed by cervical dislocation. The final body weight and tumor weight of each mouse were recorded. The change in body weight and tumor inhibition were calculated by the following equations:

Percentage change in body weight (%)

$$= \frac{\text{Average (final body weight – initial body weight)}}{\text{Average Initial body weight}} \times 100\ \%$$

7-8 weeks male BALB/c mice

↓

Acclimatize for a week

↓

Day 0

1. Anesthetize the mice by diethyl ether
2. Record the initial weight
3. Inoculate S-180 tumor cells (5×10^6 cells/0.2 ml) onto the back of mice (s.c.)

↓

Day 3 to 12 Administration of samples / PBS for 10 consecutive days (i.p.)

↓

Day 21

1. Sacrifice the mice by cervical dislocation
2. Enucleate the tumor from the mice
3. Record the tumor and final body weights

Fig. 3.3 A flow chart showing the steps for studying the *in vivo* antitumor effect of the polysaccharides.

Percentage of tumor inhibition (%)

$$= \left(1 - \frac{\text{Average tumor weight of treatment group}}{\text{Average tumor weight of control group}} \right) \times 100\%$$

A simplified flow chart illustrating the steps and procedures for studying the antitumor effect of DI3 is shown in Fig. 3.3.

3.3.4 Effect of DI3c Fraction on Tumor Necrosis Factor-Alpha (TNF-α) and Interleukin 2 (IL-2) Production (Ohno *et al.*, 1995)

3.3.4.1 Treatment of Mice

BALB/c mice (8-9 week old) were used in this experiment. They were divided randomly into four groups. One of them received 12.5 mg/kg DI3c in sterile PBS for 5 consecutive days, and another group received sterile PBS only (i.p.). After 5 days of injection, each mouse of these two groups was given 10 μg LPS (Sigma) in 0.2 ml PBS (i.v.). The other two groups received either 12.5 mg/kg DI3c or PBS only for 5 consecutive days (i.p.).

3.3.4.2 Preparation of Mouse Serum

Mouse blood was obtained by eye puncture 1 hour after LPS administration,

and was allowed to clot at 4°C for 1 hour. The collected blood was then centrifuged for 10 min at 1000 g with a Jouan A-14 centrifuge, and the supernatant was collected as serum for TNF-α and IL-2 assays, respectively.

3.3.4.3 Enzyme-linked Immunosorbent Assay (ELISA) for TNF-α Production

TNF-α in the serum sample was determined by a sandwich ELISA kit, OptEIA™ Set Mouse TNF-α (Mono/Poly) (PharMingen). All the steps and procedures were followed as those stated in the manual of the kit. In brief, 100 μl per well of anti-mouse TNF-α monoclonal antibody diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) was bound to the surface of 96 well, flat bottom plates (96-well Nunc Maxisorp) by incubating at 4°C overnight. The plate was aspirated and washed for 5 times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (washing buffer), and blocked with 200 μl assay diluent (provided) at room temperature for 1 hour. After 5 times of aspirating and washing with washing buffer, the plate was sealed and incubated with 100 μl diluted recombinant mouse TNF-α in assay diluent or samples at room temperature for 2 hours. The plate was then washed 5 times with washing buffer, and was then treated with 100 μl diluted biotinylated anti-mouse TNF-α polyclonal antibody in assay diluent. After 1 hour of incubation, the plate was aspirated and washed 5 times with washing buffer, which

was then treated with 100 μl diluted enzyme reagent (avidin-horseradish peroxidase conjugate). After washing the wells for 7 times with the washing buffer to remove any unbound antibody, presence of the peroxidase-conjugated antibody was detected by the addition of 100 μl TMB (3,3',5,5'-tetramethylbenzidine) substrate reagent (provided), and incubated for 30 min at room temperature. Color development was stopped by the addition of 50 μl 2 N sulfuric acid, and the absorbance of each well at 450 nm was determined by the microplate reader with a reference wavelength of at 570 nm. A standard calibration curve was also prepared using recombinant mouse TNF-α in this study.

3.3.4.4 Enzyme-linked Immunosorbent Assay (ELISA) for IL-2 Production

IL-2 in the serum sample was determined by a sandwich ELISA kit, OptEIA™ Set Mouse IL-2 (Mono/Poly) (PharMingen). All the steps and procedures were followed as those stated in the manual of the kit. In brief, 100 μl per well of anti-mouse IL-2 monoclonal antibody diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) was bound to the surface of 96 well, flat bottom plates (96-well Nunc Maxisorp) by incubating at 4°C overnight. The plate was aspirated and washed for 5 times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (washing buffer), and blocked with 200 μl assay diluent (provided) at room

temperature for 1 hour. After 5 times of aspirating and washing with washing buffer, the plate was sealed and incubated with 100 μl diluted recombinant mouse IL-2 in assay diluent or samples at room temperature for 2 hours. The plate was then washed 5 times with washing buffer, and was then treated with 100 μl diluted biotinylated anti-mouse TNF-α polyclonal antibody in assay diluent. After 1 hour of incubation, the plate was aspirated and washed 5 times with washing buffer, which was then treated with 100 μl diluted enzyme reagent (avidin-horseradish peroxidase conjugate). After washing the wells for 7 times with the washing buffer to remove any unbound antibody, presence of the peroxidase-conjugated antibody was detected by the addition of 100 μl TMB (3,3',5,5'-tetramethylbenzidine) substrate reagent (provided), and incubated for 30 min at room temperature. Color development was stopped by the addition of 50 μl 2 N sulfuric acid, and the absorbance of each well at 450 nm was determined by the microplate reader with a reference wavelength of at 570 nm. A standard calibration curve was also prepared using recombinant mouse IL-2 in this study.

3.4 *In vitro* Studies

3.4.1 Maintenance of Cell Lines

Three human cell lines (acute promyelocytic leukemia HL-60, chronic myelogenous leukemia K-562 and liver cancer HepG2) and one murine Sarcoma-180 (S-180) cell line were used to study the antiproliferative and cytotoxic effect of DI3 fraction in this study. All the cell lines were provided by American Type Culture Collection (ATCC) (Rockville, MD). The human cell lines were grown, in RPMI 1640 medium (Sigma), supplemented with 0.2% sodium bicarbonate (Sigma), 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml amphotericin B (all from GIBCOBRL). The S-180 cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCOBRL), supplemented with 0.37% sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml amphotericin B. A monkey kidney Vero cell line was also used in the toxicity test, which was also provided by ATCC. The cells were grown in Minimal Essential Medium (MEM) (GIBCOBRL), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml amphotericin B in the presence of 20 mM Hepes, 2 mM of glutamine and 0.024 mg/ml gentamycin. All the cells were maintained under a fully humidified atmosphere of 95% room air and 5%

CO_2 at 37°C in 25 cm^2 sterile polystyrene tissue culture flasks (Sarstedt). The medium was replaced three times a week, and cell density and viability were routinely monitored by hemocytometer and Trypan Blue exclusion method (detailed in Section 3.4.4).

3.4.2 Cytotoxicity on Cancer Cell Lines

HL-60, K-562 and S-180 cells were seeded, especially, in 96-well round bottom culture plates (IWAKI) at an initial density of 5×10^3 cells per 100 µl (i.e. 5×10^4 cells/ml), HepG2 cells were seeded in 96-well flat bottom culture plates (IWAKI) at a an initial density of 2×10^4 cells per 200 µl (i.e. 1×10^5 cells/ml). Each of cells was incubated, respectively, with 100, 200, 400 and 800 µg/ml of DI3 fractions for 72 hours. The cytotoxic effects of DI3 fraction on HL-60, K-562 and S-180 cells were determined by Trypan blue exclusion assay. The cytotoxic effects of DI3 fractions on HepG2 cells were measured by MTT assay.

3.4.3 Cytotoxicity on Normal Cell Line

Vero cells were seeded in a 96-well flat bottom culture plate at an initial density of 2×10^4 cells per 200 µl (i.e. 1×10^5 cells/ml). DI3 fraction at concentrations of 250, 500 and 1000 µg/ml were incubated, respectively, with the cells for 72 hours.

The toxicity was measured by MTT assay.

3.4.4 Trypan Blue Exclusion Method

Effect of DI3 fraction on proliferation of HL-60, K-562 and S-180 were determined by counting the cell number with hemocytometer. In meanwhile, the cell viability was examined by Trypan blue exclusion method. After incubating with different concentrations of DI3 fractions, 100 μl of 0.4% Trypan blue solution in PBS (w/v) was added to each well. The total number of cells and the trypan blue-stained cells in the four 1-mm^2 corners of the hemocytometer were counted. The cell density and viability were calculated by the following equations:

$$\text{Cell density} = \frac{\text{The total cell number in the four 1-mm}^2\text{ corners}}{4} \times \text{Dilution factor} \times 10^4 \text{ cells/ml}$$

$$\text{Viability (\%)} = \frac{\text{Viable cell count}}{\text{Total cell count}} \times 100\%$$

In order to determine the IC_{50} of DI3 fractions on different cancer cell lines, the percentage of inhibition was also expressed by following equation:

$$\left(1- \frac{\text{Average cell density in treatment group}}{\text{Average cell density in control group}}\right) \times 100\%$$

3.4.5 MTT Assay

The cytotoxic effects of DI3 fraction on HepG2 and Vero cell lines were determined by MTT assay (Mosmann, 1983). After incubating with DI3 fractions, MTT solution was freshly prepared by dissolving MTT (3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) in filtered PBS at 5 mg/ml. 20 μl of the MTT solution was added to each well. After 5 hours of incubation, the medium was removed and the blue formazan formed was dissolved by adding 150 μl of acidic isopropanol (0.04 N HCl). All the wells were then mixed by pipetting until all crystals were dissolved completely. Absorbance of the blue formazan solution generated was measured at 570 nm by the microplate reader. The percentage of inhibition was expressed by the following equation:

$$\left(1- \frac{\text{Average absorbance of treatment group}}{\text{Average absorbance of control group}}\right) \times 100\%$$

3.4.6 BrdU Incorporation (Takeyoshi *et al.*, 2001)

Besides counting the cell number, effect of DI3b fraction on proliferation of HL-60 cells was further determined using the cell proliferation assay kit (Roche). HL-60 cells were seeded in a 96-well flat-blottom microplate at 5×10^4 cells/ml, and were incubated with 100, 200, 400 and 800 μg/ml of DI3 fractions, respectively, for 72 hours. After incubation of 10 μl BrdU labeling solution (provided) was added to each well and incubated at 37°C for 2 h. After centrifugation by Centra MP4R (IEC) at 300 g for 10 min, the supernatant were removed, and the plate was aspirated for 15 min. 200 μl of Fix-Denat solution (provided) was added to each well to fix the cells, and the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denant solution, the diluted anti-BrdU-POD working solution (provided) was added to each well and incubated for 90 min. After rinsing three times with the provided washing buffer, 100 μl of substrate solution was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader at a reference wavelength at 492 nm.

3.5 Statistical Analysis

Results were expressed as mean ± standard deviation (S.D.) and analyzed by Student's *t*-test. The level of significance was taken at $p<0.05$.

Chapter 4

Results

4.1 Extraction and Fractionation of Polysaccharides from *Dicytophora indusiata*

4.1.1 Percentage Yield of Crude DI Polysaccharides

After hot water extraction, 3.1 g DI2 and 10.1 g DI3 were obtained from 200 g of dried fruit bodies of mushroom *Dicytophora indusiata* with 40% and 80% ethanol precipitation, respectively. The total percentage yield of crude DI polysaccharides was 6.6 % (Table 4.1).

4.1.2 Percentage Yield of DI3 Fractions

To further fractionate crude DI3 polysaccharides, 50 mg of DI3 was applied onto a DEAE-cellulose column. After eluted by distilled water, 0.04 M and 0.5 M NaCl, polysaccharides fractions designated as DI3a (34.7 mg), DI3b (4.6 mg) and DI3c (5.5 mg) were obtained, respectively (Fig. 4.1). Recovery of the DI3 fractions

after anion exchange chromatography was 89.6% (Table 4.2).

	Weight (g)	Yield (%)
Dried fruit bodies	200	---
DI2	3.1	1.55
DI3	10.1	5.05

Table. 4.1 Yields of different polysaccharide fractions from 200 g of dried fruit bodies of *Dictyophora indusiata*.
DI2 polysaccharides were obtained from ethanol precipitation up to 40% final concentration.
DI3 polysaccharides were obtained from ethanol precipitation up to 80% final concentration.

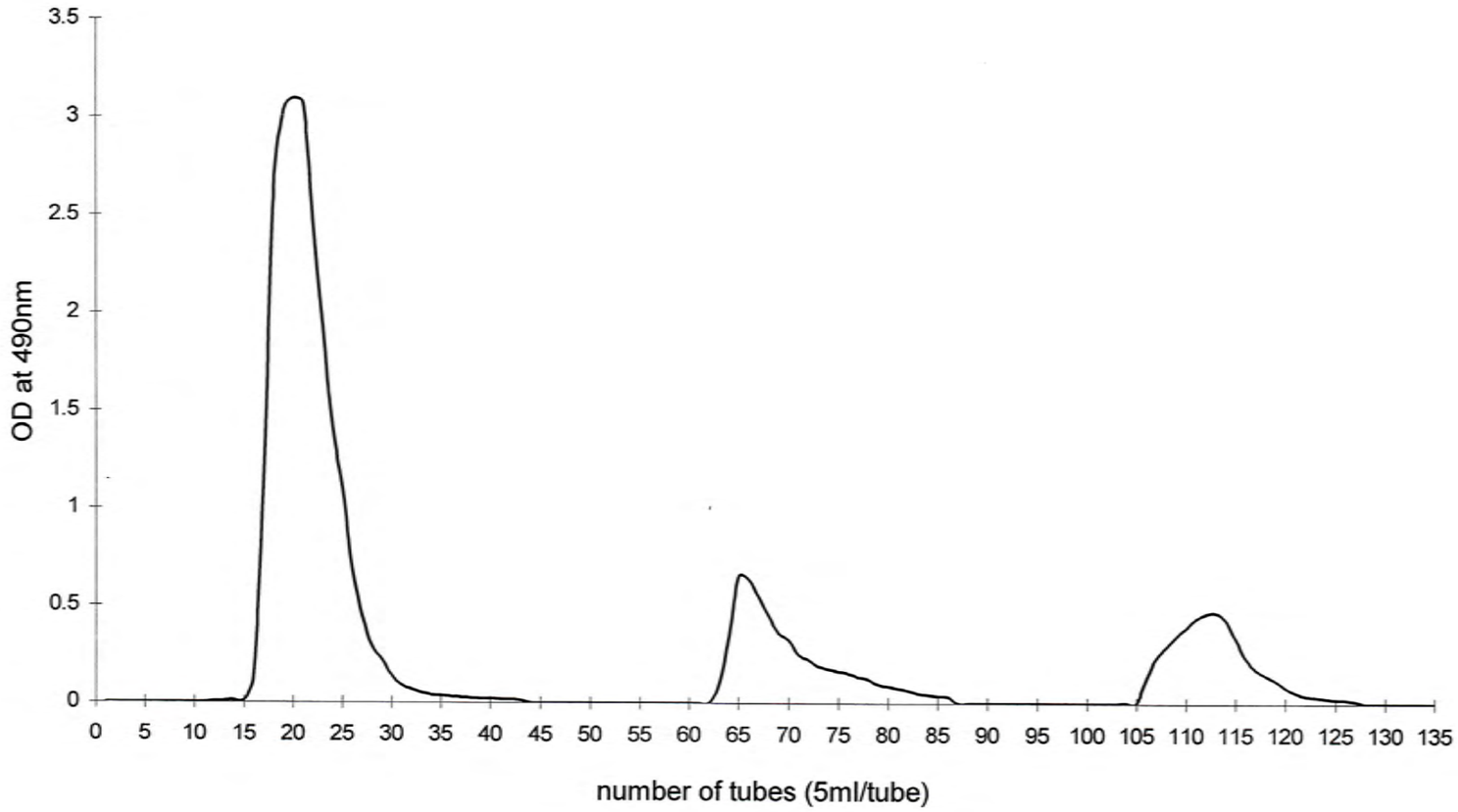

Fig. 4.1 Anion exchange chromatography of DI3 crude polysaccharides on a DEAE-cellulose column.
50mg of DI3 was applied to the column. DI3a, DI3a and DI3c fractions were eluted by distilled water, 0.04 M and 0.5 M NaCl, respectively. Fractions of 5ml/ tube were collection at a flow rate of 20ml/hr.
The presence of polysaccharides was determined by phenol-sulfuric assay.

	Weight (mg)	Yield (%)
DI3	50	---
DI3a	34.7	69.4
DI3b	4.6	9.2
DI3c	5.5	11.0
Recovery	44.8	89.6

Table. 4.2 Yields of different polysaccharide fractions from 50 mg of DI3 polysaccharide fraction in DEAE-cellulose column.
DI3 polysaccharides were obtained from ethanol precipitation up to 80% final concentration.
DI3a, DI3b and DI3c fractions were eluted by distilled water, 0.04 M and 0.5 M NaCl solution, respectively, from the DEAE-cellulose column.
The collected freeze-dried DI3 fractions were weighed after desalting by dialysis.

$$\text{Recovery (\%)} = \frac{\text{Total weight of DI3 fractions}}{\text{Weight of crude DI3 polysaccharides}} \times 100\ \%$$

4.2 Characterization of DI3 Fractions

4.2.1 Polysaccharide and Protein Contents of DI3 Fractions

Contents of polysaccharides and proteins were determined by phenol sulfuric acid assay and Lowry-Folin method, respectively (Table 4.3). Each mg of the crude DI3 polysaccharides contained 0.64 mg of polysaccharides and 0.12 mg of proteins. Among the three fractions, DI3a contained the highest amount of polysaccharides (0.94 mg per mg of sample), and DI3c contained the highest amount of proteins (0.25 mg per mg of sample).

4.2.2 Relative Monosaccharide and Uronic Acid Content in Different DI3 Fractions

The relative monosaccharide and uronic acid content in different DI3 fractions were determined by gas chromatography and colorimetric method, respectively. As shown in Table 4.4, DI3 fractions were mainly composed of monosaccharide, including mannose, glucose, galactose and uronic acid. Trace amounts of xylose were also found in DI3, DI3b and DI3c fractions. Among the three DI3 fractions, mannose constituted the highest amount in DI3a (16.84 %) and DI3b (19.96 %) fractions, while glucose constituted the highest amount in DI3c fractions (19.31 %).

Methods used	Lowry-Folin method	Phenol-sulfuric assay
	Proteins contents (%)	Polysaccharides contents (%)
DI3	12 ± 0.6	64 ± 2
DI3a	3 ± 0.3	94 ± 2
DI3b	10 ± 0.6	88 ± 4
DI3c	25 ± 0.5	72 ± 1

Table. 4.3 Protein and polysaccharide contents in DI3 polysaccharides and different DI3 fractions.
Protein and polysaccharide contents were determined by Lowry-Folin method and Phenol-sulfuric assay, respectively.
Results are expressed as mean ± S.D. (n=3)

Monosaccharides	Proportions (% w / w) [a]			
	DI3	DI3a	DI3b	DI3c
Fucose	N.D.	N.D.	N.D.	N.D.
Galactose	1.03	1.72	1.70	0.89
Glucose	7.86	4.71	10.40	19.31
Mannose	13.34	16.84	19.96	4.19
Xylose	T	N.D.	T	T
Arabinose	N.D.	N.D.	N.D.	N.D.
Ribose	N.D.	N.D.	N.D.	N.D.
Rhamnose	N.D.	N.D.	N.D.	N.D.
Uronic acid	2.75	0.03	1.21	17.09

Table. 4.4 Proportions of different monosaccharides and uronic acid in crude DI3 polysaccharides and different DI3 fractions determined by gas chromatograpy.

[a] Results are expressed as relative proportions (% w/w) in each sample.
"N.D." indicates that the particular monosaccharide was not detectable.
"T" indicates that only trace amount of monosaccharide was detected.

4.2.3 Estimated Molecular Weight of DI3 Fractions

Gel permeations of the DI3a, DI3b and DI3c fractions are shown in Fig. 4.2, 4.3 and 4.4, respectively. A non-polysaccharides peak was appeared in the elution curves of DI3 fractions and dextran standards, at a retention time of about 25.5 min. The molecular weight of different compounds in different DI3 fractions were determined by measuring their retention times and compared them to the dextran standard (Fig. 4.5).

DI3c is a relatively homogenous polysaccharide fraction, and the average molecular weight was about 3.3 kDa (Fig. 4.4). Both DI3a and DI3b fraction contained more than one group of polysaccharides. DI3a fraction contained two groups of polysaccharides and the average molecular weight were determined to be 17 kDa and 284 kDa, respectively (Fig. 4.2). DI3b fraction contained three groups of polysaccharides, and their average molecular weights were 2.5 kDa, 12 kDa and >670 kDa, respectively (Fig.4.3).

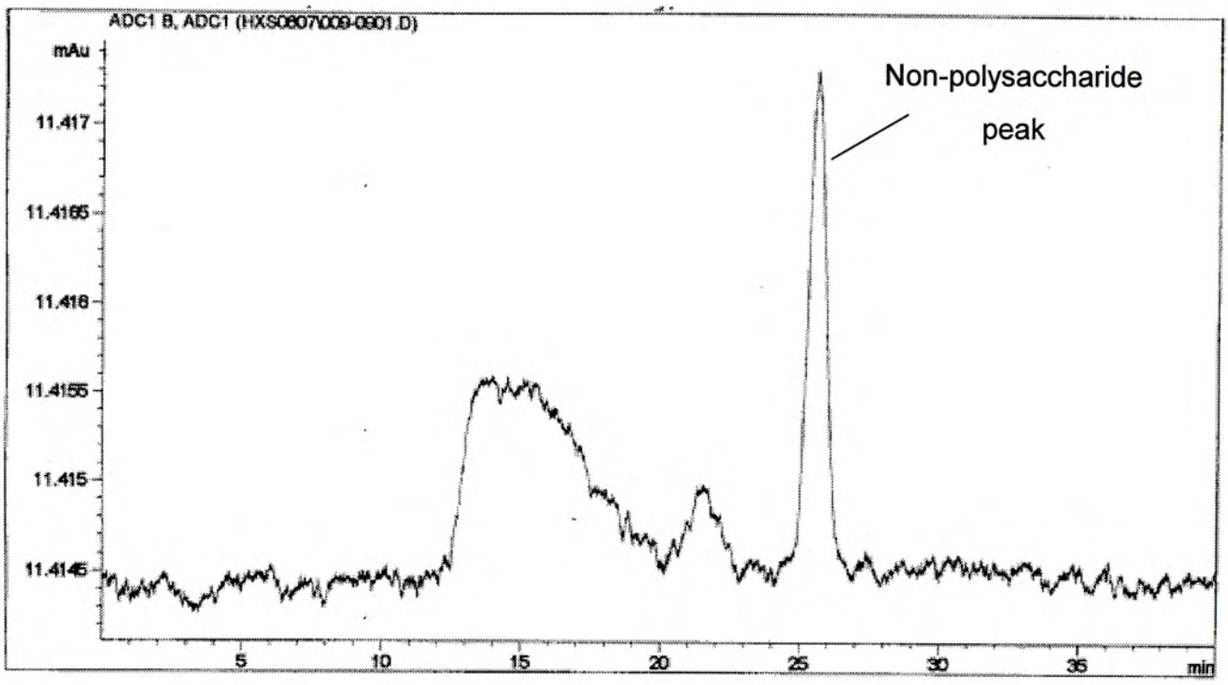

Fig. 4.2 Gel permeation of DI3a fraction.

Molecular weight of DI3a fraction was determined by HPLC using dextran standards with molecular weight of 1, 5, 25, 80, 270 and 670 kDa.

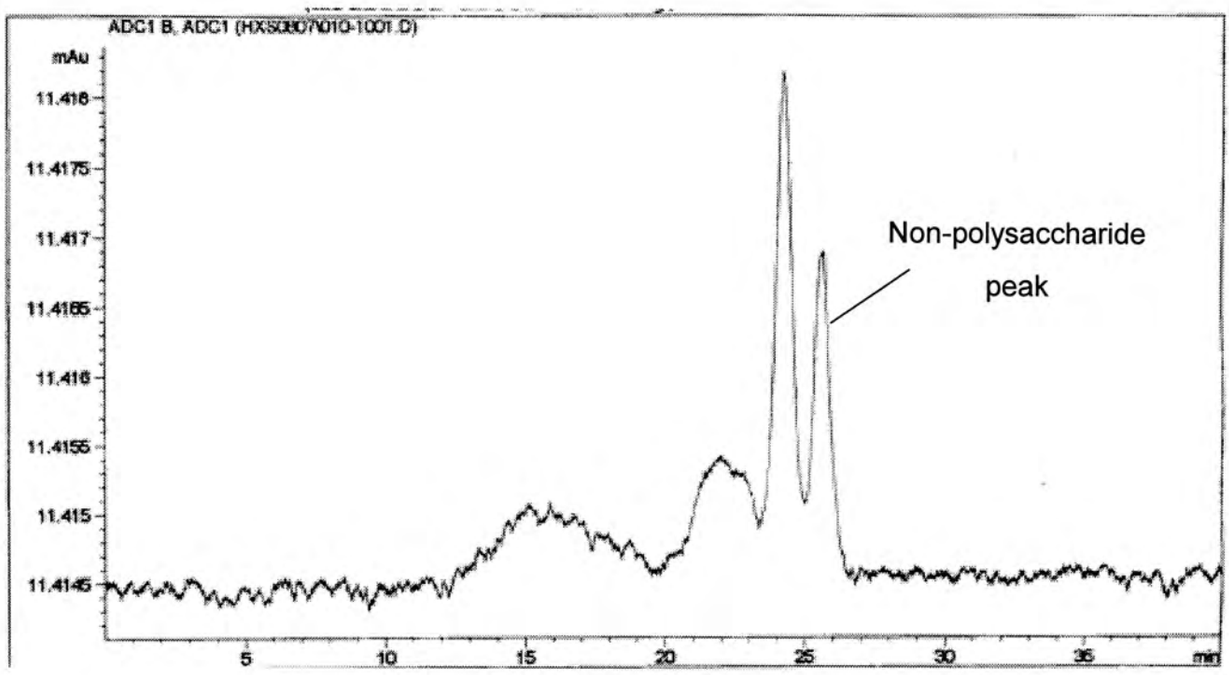

Fig. 4.3 Gel permeation of DI3b fraction.

Molecular weight of DI3b fraction was determined by HPLC using dextran standards with molecular weight of 1, 5, 25, 80, 270 and 670 kDa.

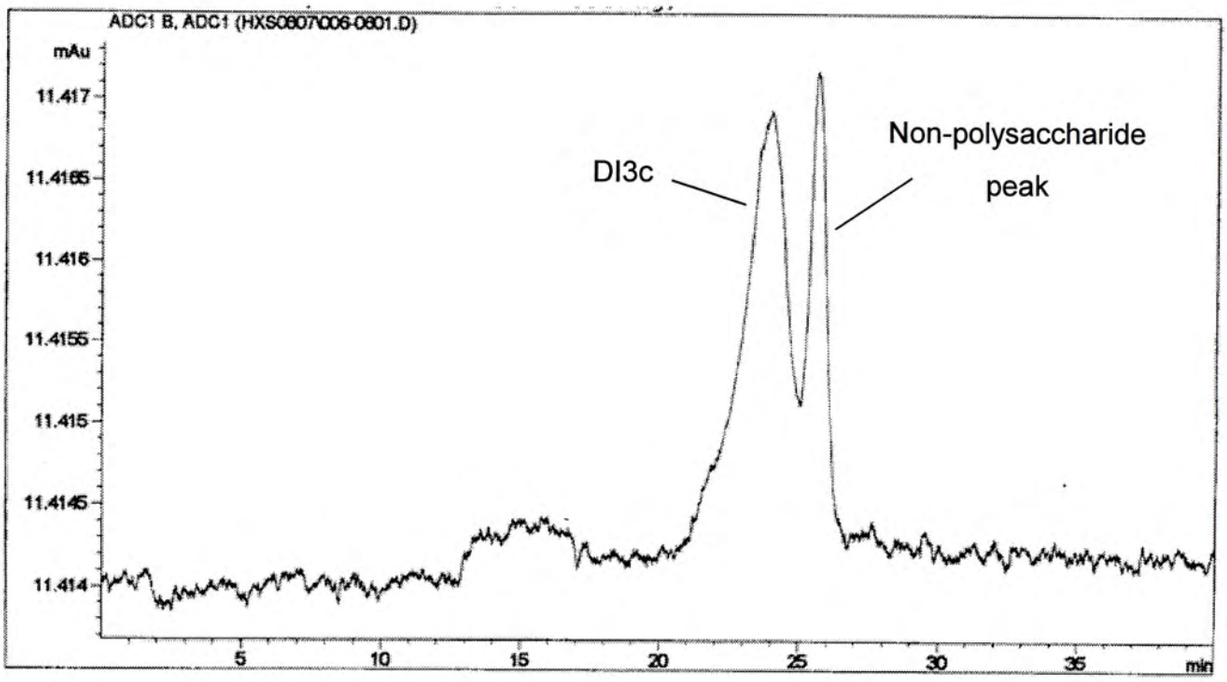

Fig. 4.4 Gel permeation of DI3c fraction by HPLC.

Molecular weight of DI3c fraction was determined by HPLC using dextran standards with molecular weight of 1, 5, 25, 80, 270 and 670 kDa.

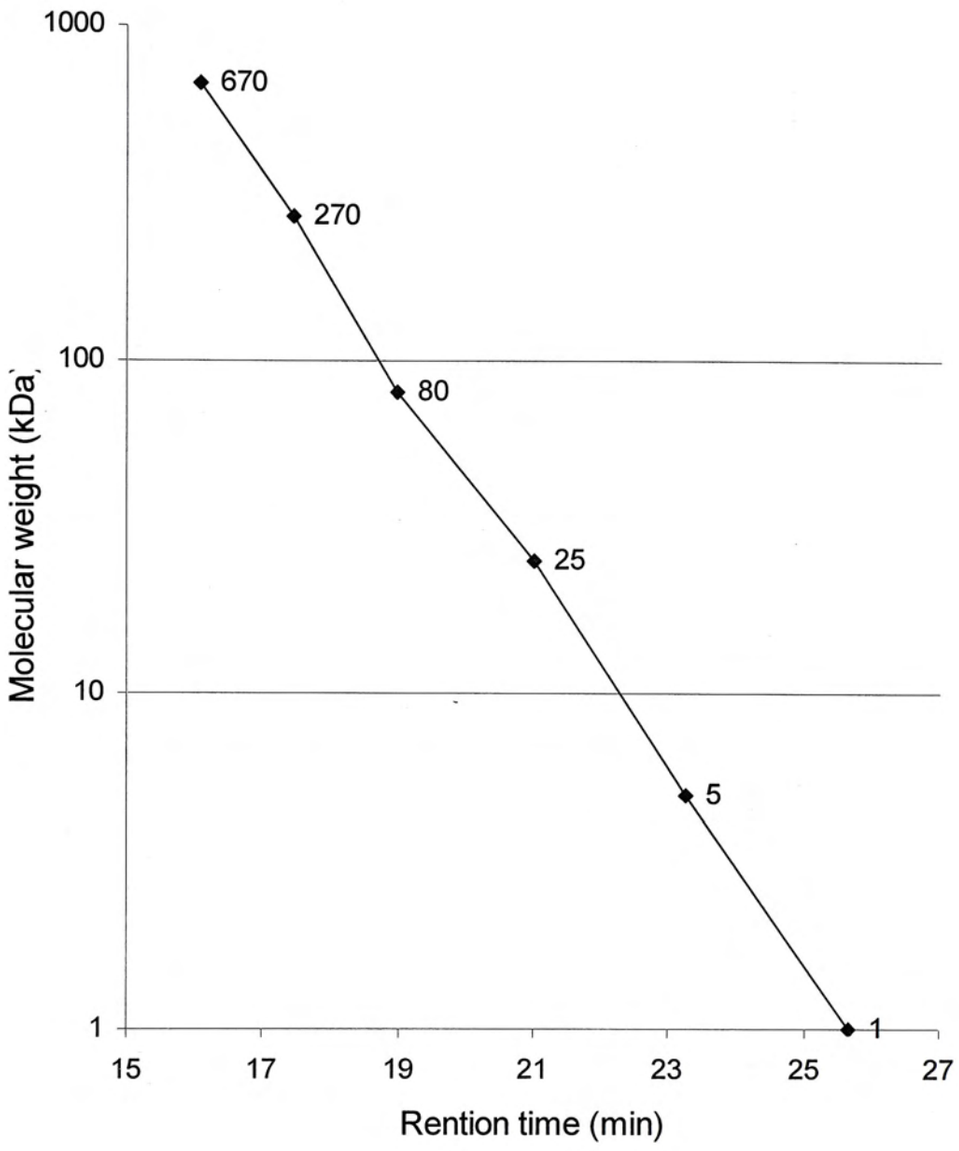

Fig. 4.5 HPLC determinations of molecular weights of different DI3 fractions using dextran standards of 1, 5, 25, 80, 270 and 670 kDa.

4.3 Antitumor Effect of *Dictyophora indusiata* polysaccharides *In vivo*

4.3.1 *In vivo* Antitumor Effect of Crude DI Polysaccharides

The crude DI polysaccharides were administered to Sarcoma-180 solid tumor-bearing mice to study the *in vivo* antitumor activity. Both DI2 and DI3 fractions were administered, respectively, 72 h after the tumor implantation, and were then given a suitable dose of sample for 10 consecutive days. It was found that only DI3 fraction could reduce the tumor weight significantly by 64% (Fig. 4.6).

4.3.2 *In vivo* Antitumor Effect of Various Fractions of DI3

All the fractions derived from DI3 reduced the weight of solid tumor. At a dose of 20 mg/kg, DI3c showed the highest reduction, and the tumor weight was reduced by 59%; whereas, DI3b reduced the weight by 51 % (Fig. 4.7).

When crude DI3 polysaccharides and DI3c fraction were administered into tumor-bearing mice at different doses, both of them reduced the tumor weight dose-dependently. At 50 mg/kg, the effect of DI3c was more prominent than that of DI3. DI3c reduced the tumor weight by 76% (Fig. 4.9), whereas DI3 reduced the tumor weight by 53% of the control level (Fig. 4.8).

Neither crude DI3 polysaccharides nor DI3c fraction could significantly affected the body weight of tumor-bearing mice when compared to the weight control group

(normal mice) (Table 4.5 and Table 4.6).

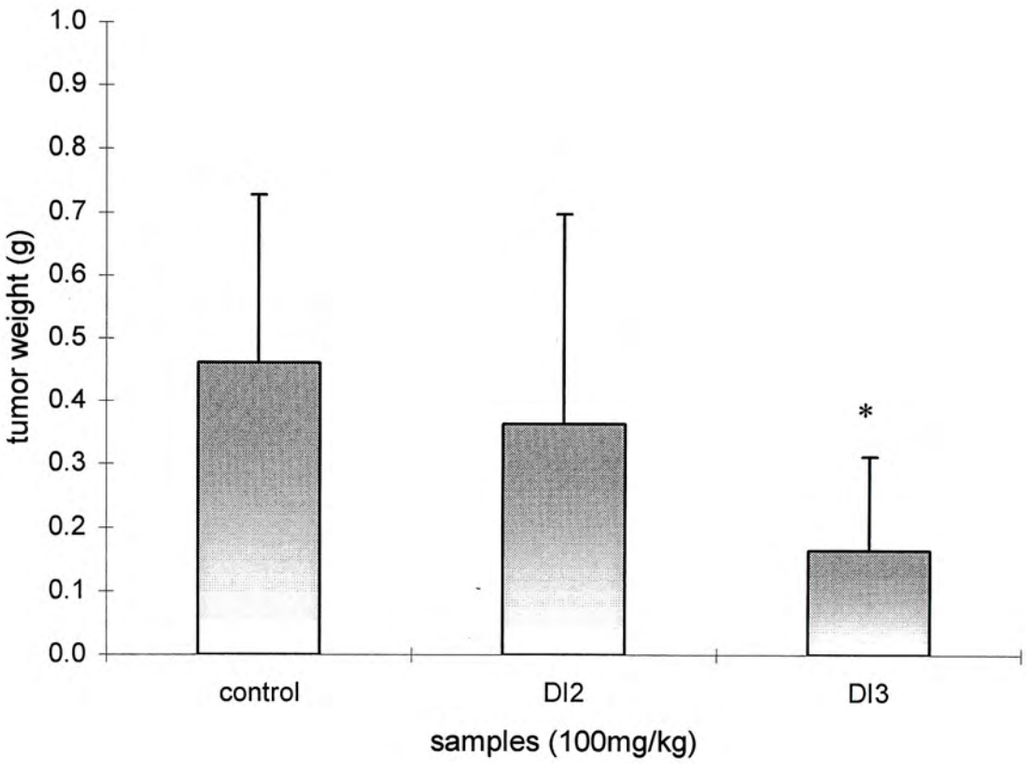

Fig. 4.6 Effect of different DI fractions on tumor weight of Sarcoma 180 solid tumor-bearing mice.

Three groups of mice received a dose of PBS, DI2 and DI3 fractions, respectively, for 10 consecutive days, 72 h after tumor implantation.

The solid tumors were enucleated and weighed at 21 days after tumor inoculation.

Results are expressed as mean (g) ± S.D. (n=7)

* represents statistical significance at $p<0.05$ when compared to the control level by Student *t*-test.

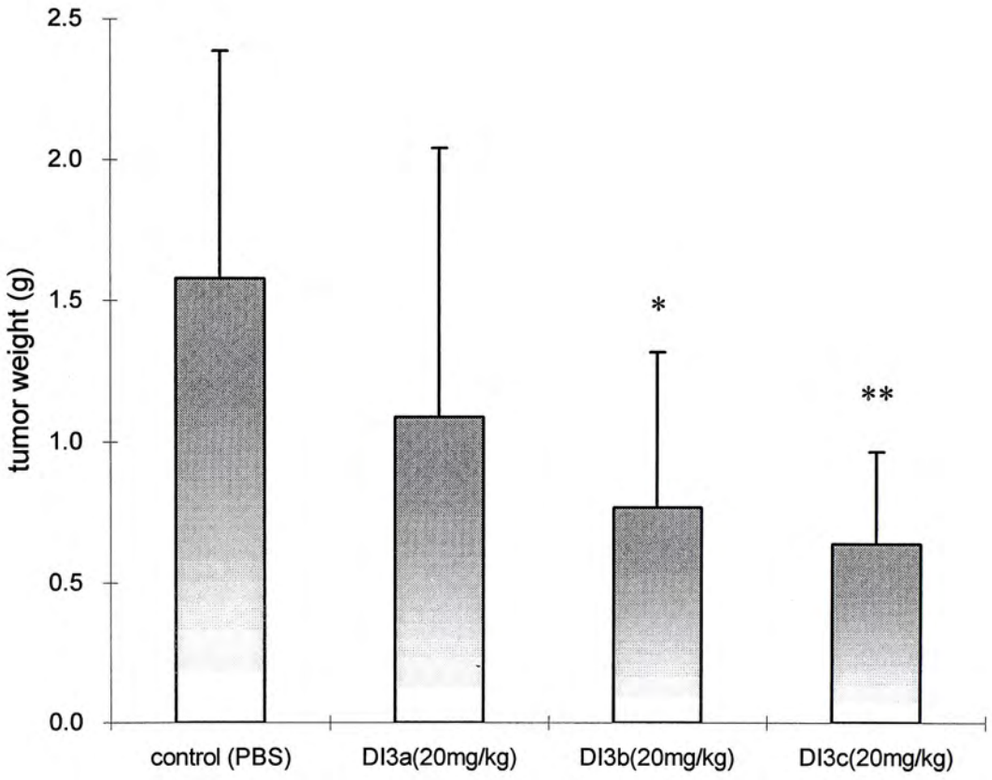

Fig. 4.7 Effect of different DI3 fractions on tumor weight of Sarcoma 180 solid tumor-bearing mice.

Four groups of mice were administered with PBS, DI3a, DI3b and DI3c fractions, respectively, for 10 consecutive days, 72 h after tumor implantation.

The solid tumors were enucleated and weighed at 21 days after tumor inoculation.

Results are expressed as mean (g) ± S.D. (n=7)

* and ** represent statistical significance at $p<0.05$ and $p<0.01$, respectively, when compared to the control level by Student *t*-test.

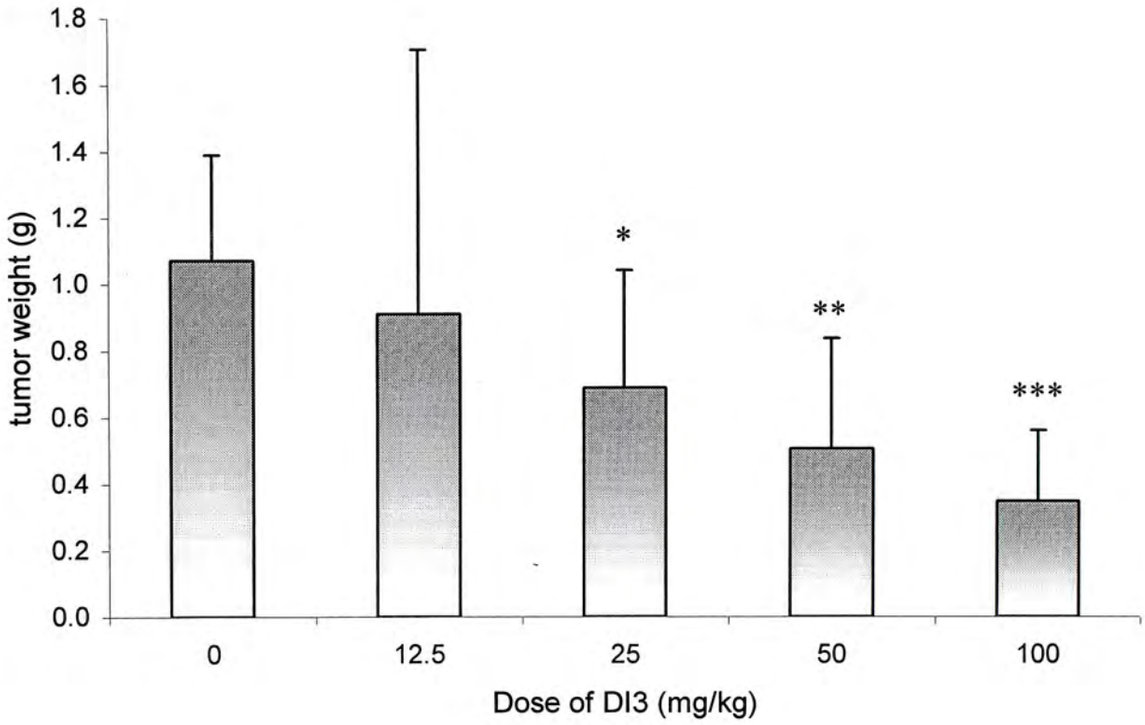

Fig. 4.8 Effect of different doses of crude DI3 polysaccharides on tumor weight of Sarcoma 180 solid tumor-bearing mice.

Five groups of mice were administered with 0 (PBS alone), 12.5, 25, 50 and 100 mg/kg of crude DI3 polysaccharides, respectively, for 10 consecutive days, 72 h after tumor implantation.

The solid tumors were enucleated and weighed at 21 days after tumor inoculation.

Results are expressed as mean (g) ± S.D. (n=8)

*, ** and *** represent statistical significance at $p<0.05$, $p<0.01$ and $p<0.005$, respectively, when compared to the control level by Student *t*-test.

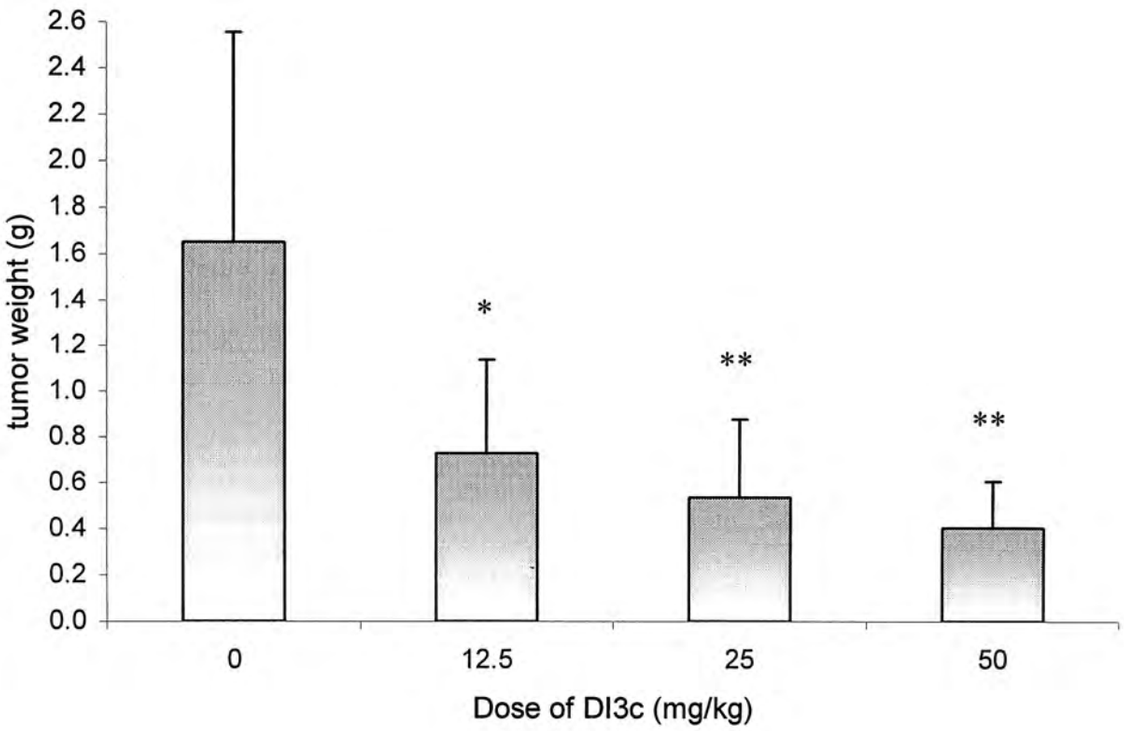

Fig. 4.9 Effect of different doses of DI3c fraction on tumor weight of Sarcoma 180 solid tumor-bearing mice.

Four groups of mice was administered with 0 (PBS alone), 12.5, 25, 50 mg/kg DI3c fraction, respectively, for 10 consecutive days, 72 h after tumor implantation.

The solid tumors were enucleated and weighed at 21 days after tumor inoculation.

Results are expressed as mean (g) ± S.D. (n=8)

* and ** represent statistical significance at $p<0.05$ and $p<0.01$, respectively, when compared to the control level by Student *t*-test

Dose (mg/kg)		Average body weight (g) ± S.D. [d]	
		Initial	Final
Weight control [a]		24.53 ± 0.82	25.52 ± 0.58
Control [b]		23.15 ± 1.29	24.02 ± 1.37
DI3 [c]	12.5	24.07 ± 1.23	24.57 ± 1.56
	25	23.93 ± 1.09	24.69 ± 1.30
	50	23.62 ± 0.81	24.61 ± 0.69
	100	23.20 ± 0.82	24.25 ± 0.98

Table. 4.5 Effect of different doses of crude DI3 polysaccharides on body weight of tumor-bearing mice.

[a] The group of mice not implanted with tumor was served as the weight control.

[b & c] The tumor bearing mice were given either PBS in control group or crude DI3 polysaccharides at different doses for 10 consecutive days.

[d] The initial body weight was measured before the tumor inoculation. The final body weight was also determined after the enucleation of the tumor from the mice.

Results are expressed as mean (g) ± S.D. (n=8)

Dose (mg/kg)		Average body weight (g) ± S.D. [d]	
		Initial	Final
Weight control [a]		23.61 ± 0.79	25.10 ± 0.97
Control [b]		23.45 ± 1.95	24.35 ± 1.89
DI3c [c]	12.5	23.74 ± 1.28	24.66 ± 1.14
	25	23.96 ± 1.05	24.92 ± 1.25
	50	23.97 ± 1.39	25.41 ± 1.37

Table. 4.6 Effect of different doses of DI3c fraction on body weight of tumor-bearing mice.

[a] The group of mice not implanted with tumor was served as the weight control.

[b & c] The tumor-bearing mice were given either PBS alone in control group or DI3c fraction at different doses for 10 consecutive days.

[d] The initial body weight was measured before the tumor inoculation. The final body weight was also determined after the enucleation of the tumor from the mice.

Results are expressed as mean (g) ± S.D. (n=8)

4.3.3 Effects of DI3c on TNF-α and IL-2 Productions in Mice

Levels of TNF-α and IL-2 in the blood of mice were measured by ELISA methods as stated in Section 3.3.4.3. Based on the previous experiment as shown in Fig. 4.8, administration of 12.5 mg/kg of DI3c reached 68% tumor reduction. And in this study, administration of 12.5 mg/kg of DI3c into the mice for 5 consecutive days in treatment groups exhibited that TNF-α levels in blood sera of DI3c treated and DI3c primed mice were significantly higher than that of the PBS control (Fig. 4.10, 4.11 and Table 4.7). DI3c fraction not only induced the production of TNF-α for about 4 folds, it also primed the LPS-triggered TNF-α production. On the other hand, the IL-2 levels in the blood sera of DI3c-treated (Fig. 4.12) and DI3c-primed mice (Fig. 4.13) did not show any significant difference from that of the PBS control group. The standard curves for determining TNF-α and IL-2 levels are shown in Fig. 4.14 and 4.15, respectively.

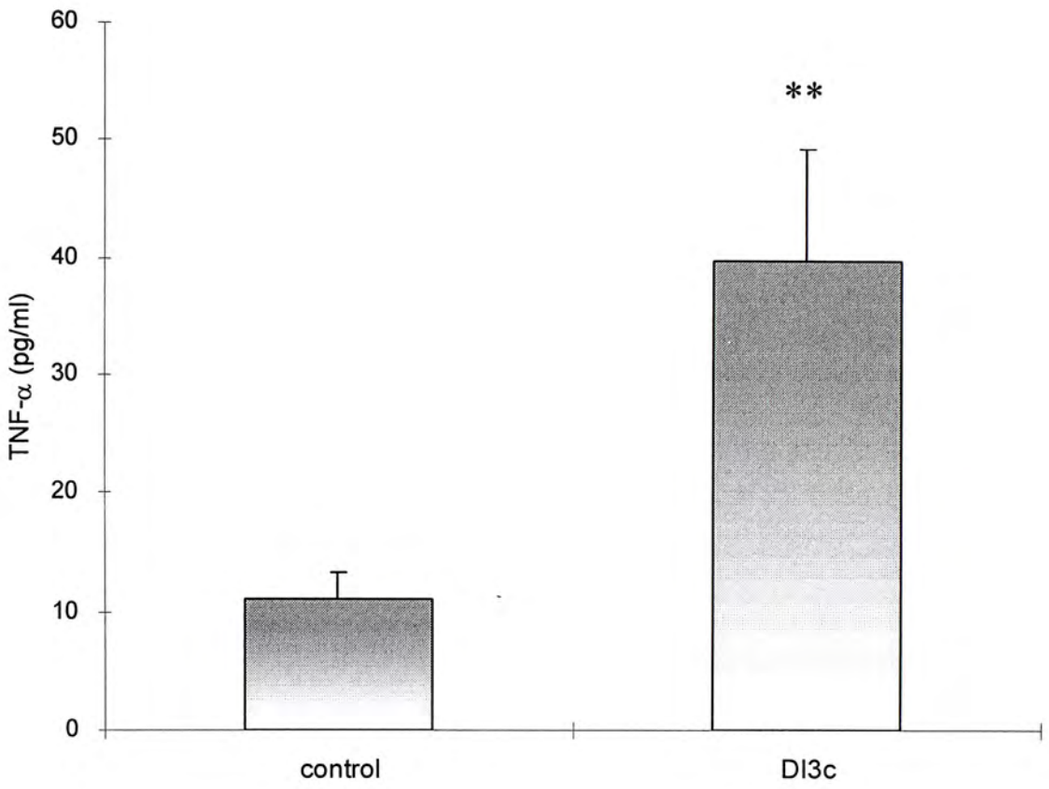

Fig.4.10 Effect of DI3c on serum level of TNF-α in mice.

TNF-α concentrations were determined by ELISA using recombinant TNF-α as a standard

Control group was given PBS only for 5 consecutive days.

The DI3c group was given DI3c fraction at a dose of 12.5 mg/kg for 5 consecutive days.

The results were expressed as mean (pg/ml) ± S.D. (n=4).

** represents statistical significance at $p < 0.01$ when compared to the control level by Student *t*-test.

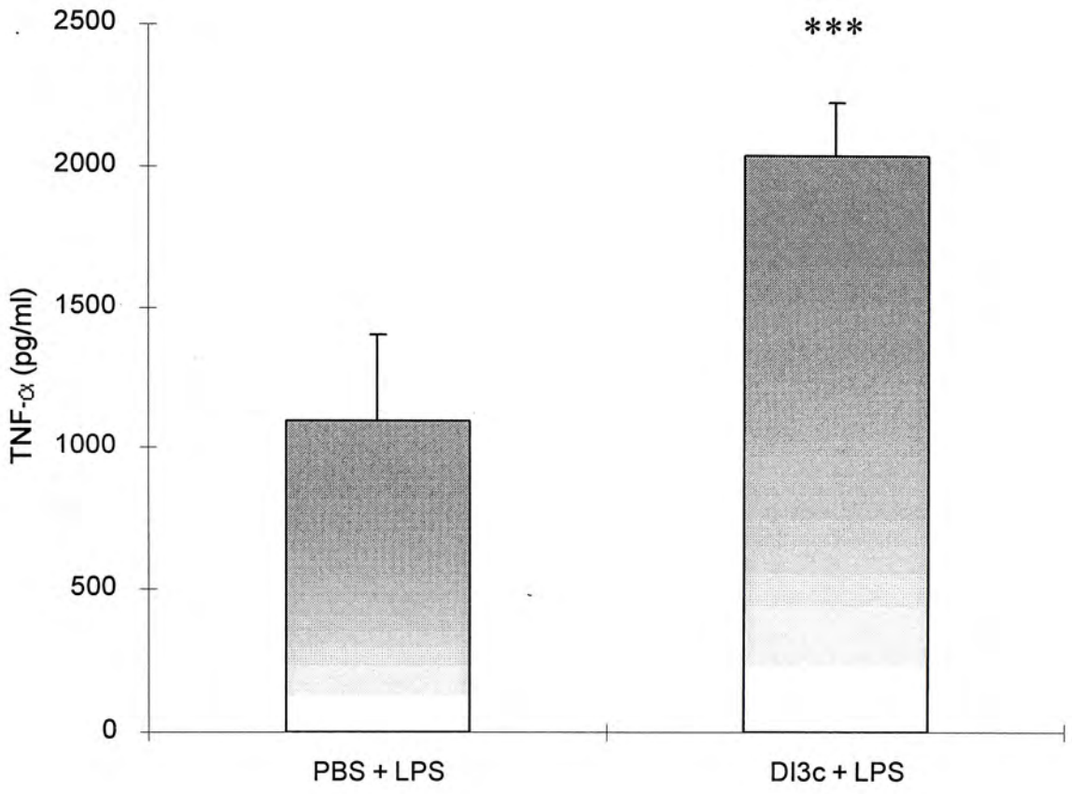

Fig.4.11 Effects of DI3c on serum level of TNF-α in LPS-primed mice.
TNF-α concentrations were determined by ELISA using recombinant TNF-α as a standard
PBS + LPS group consisted of LPS-primed mice, which had been given PBS only for 5 consecutive days.
DI3c + LPS group consisted of LPS-primed mice, which had been given DI3c fraction at a dose of 12.5 mg/kg for 5 consecutive days.
The results are expressed as mean (pg/ml) ± S.D. (n=4).
*** represents statistical significance at $p < 0.001$ when compared to the control level by Student *t*-test.

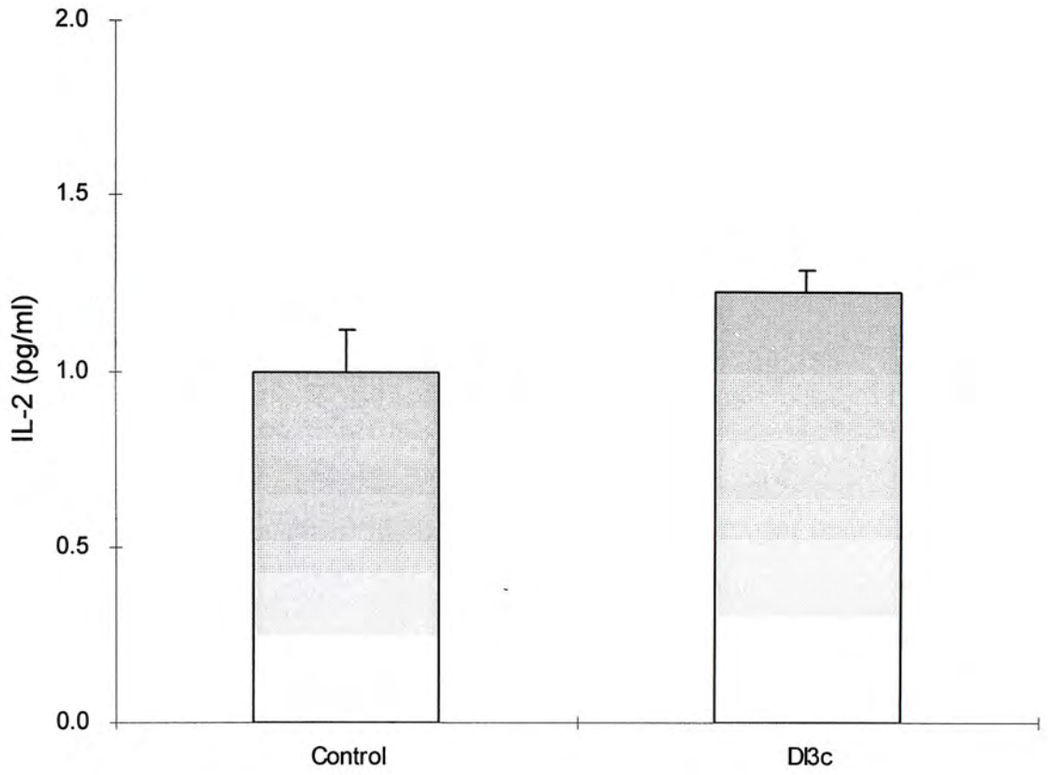

Fig.4.12 Effects of DI3c on serum level of IL-2 in mice.
IL-2 concentrations were determined by ELISA using recombinant IL-2 as a standard
Control group was given PBS only for 5 consecutive days.
The DI3c group was given DI3c fraction at a dose of 12.5 mg/kg for 5 consecutive days.
The results are expressed as mean (pg/ml) ± S.D. (n=4).

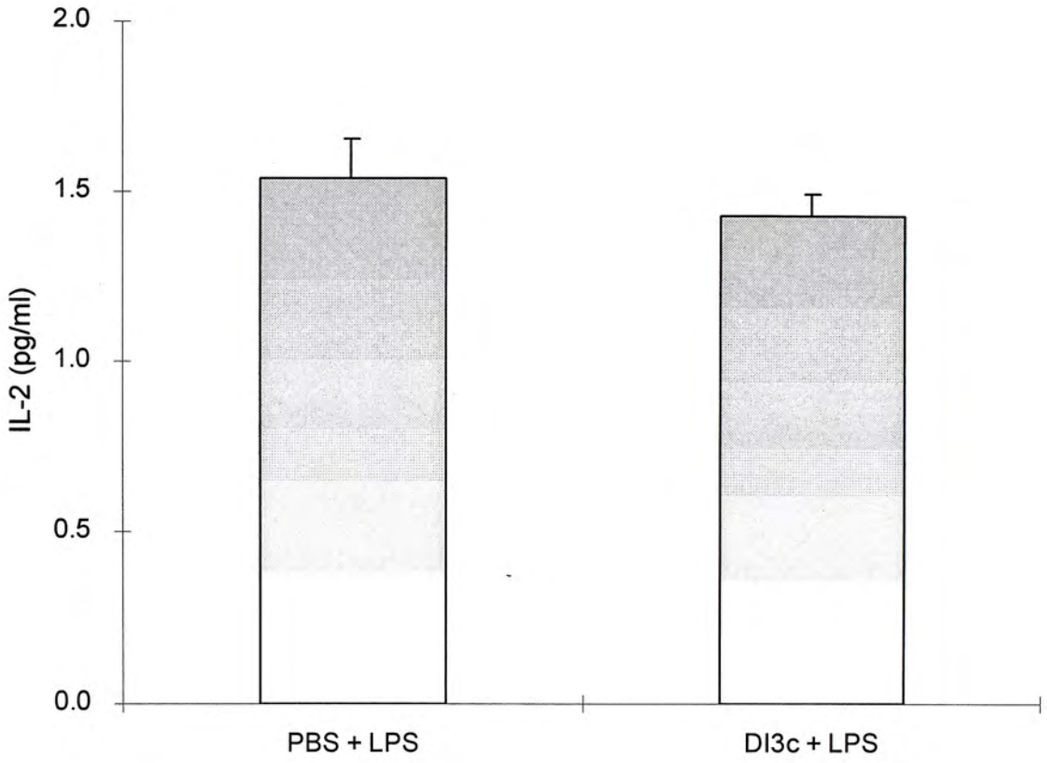

Fig.4.13 Effects of DI3c on serum level of IL-2 in LPS-primed mice.
IL-2 concentrations were determined by ELISA using recombinant IL-2 as a standard
PBS + LPS group consisted of LPS-primed mice, which had been given PBS only for 5 consecutive days.
DI3c + LPS group consisted of LPS-primed mice, which had been given DI3c fraction at a dose of 12.5 mg/kg for 5 consecutive days.
The results are expressed as mean ± S.D. (n=4).

Treatments	Serum cytokine (pg/ml)	
	TNF-α	IL-2
PBS [a]	11.11 ± 2.22	1.00 ± 0.12
DI3c [b]	39.79 ± 9.48 **	1.22 ± 0.06
PBS + LPS [c]	1093.72 ± 307.18	1.54 ± 0.59
DI3c + LPS [d]	2036.77 ± 185.25 ***	1.43 ± 0.36

Table. 4.7 Effects of DI3c on TNF-α and IL-2 production in mice.
TNF-α and IL-2 concentrations were determined by ELISA using recombinant as a standard
[a] PBS was administered i.p. to mice for 5 consecutive days
[b] DI3c (12.5 mg/kg) was administered i.p. to mice for 5 consecutive days
[c] PBS was administered i.p. to mice for 5 consecutive days before LPS (10 μg) administration
[d] DI3c (12.5 mg/kg) was administered i.p. to mice for 5 consecutive days before LPS (10 μg) administration.
The results are expressed as mean TNF-α or IL-2 concentration (pg/ml) ± S.D. of four mice.
Values that are significantly different from that of PBS control group were indicated by ** at $p < 0.01$ and *** at $p < 0.005$.

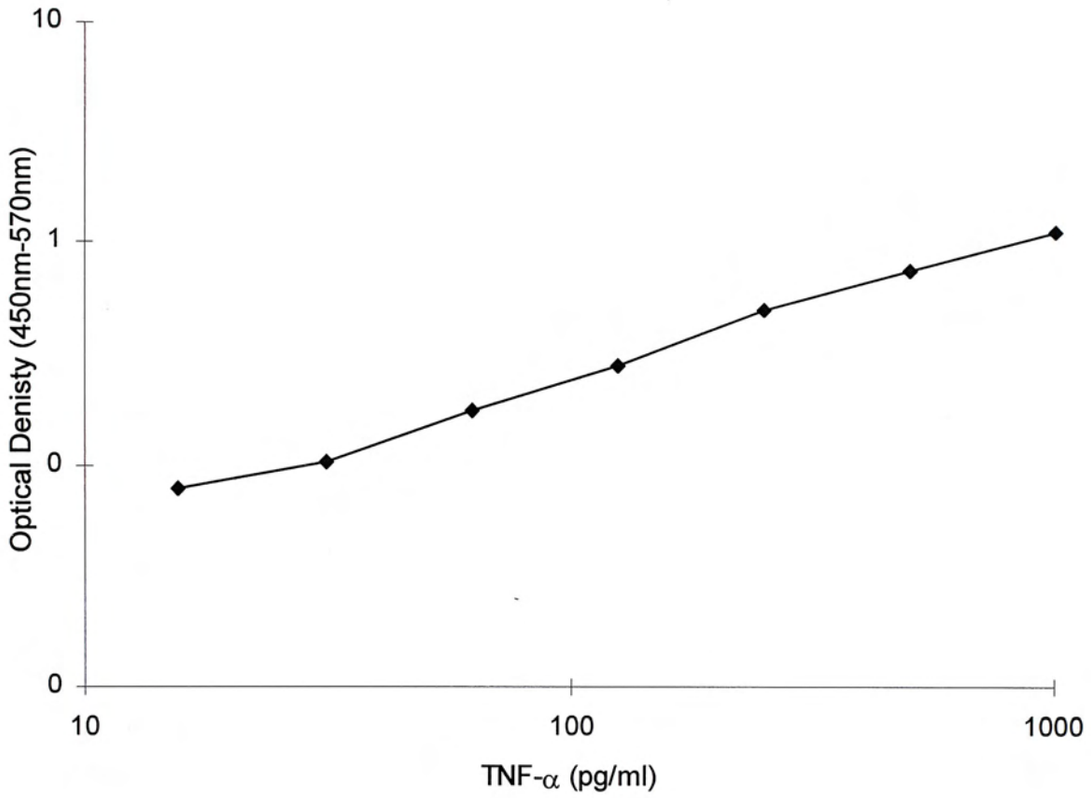

Fig. 4.14 Mouse TNF-α (Mono/Poly) standard curve.

Serial dilution of 1000 pg/ml of recombinant mouse TNF-α was used as the standard.

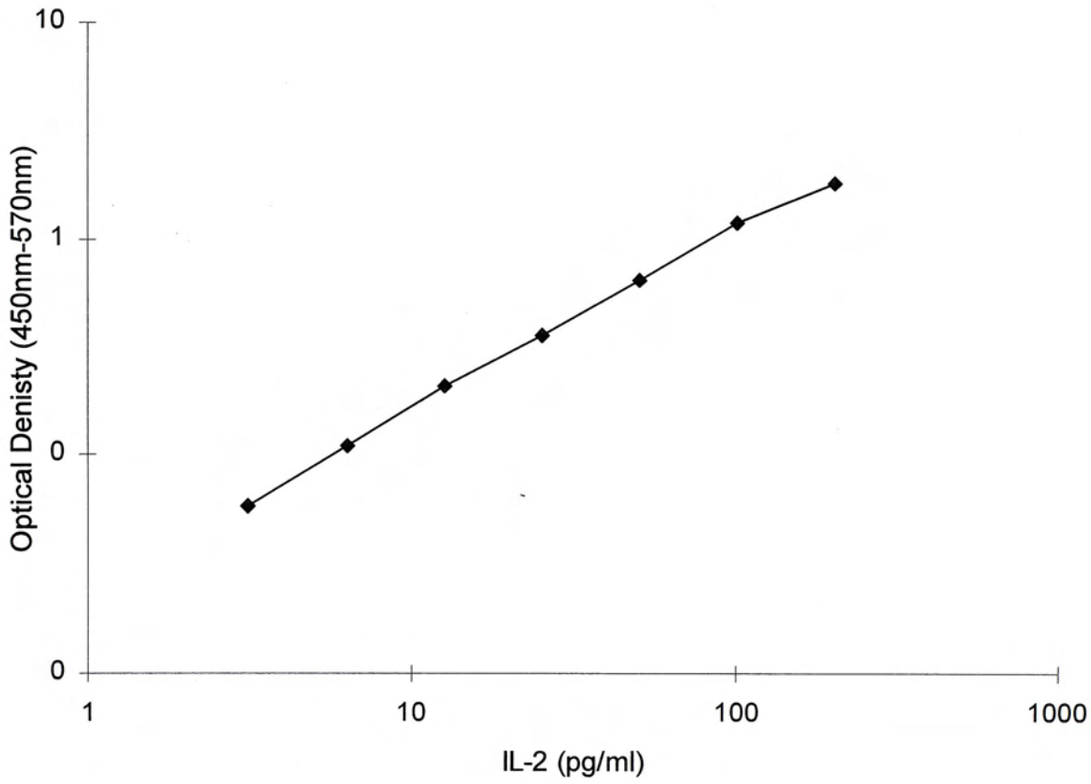

Fig. 4.15 Mouse IL-2 (Mono/Poly) standard curve.

Serial dilution of 200 pg/ml of recombinant mouse IL- 2 was used as the standard.

4.4 *In vitro* Effects of DI3 Fractions on Cell Density and Viability on Normal and Cancer Cell Lines

4.4.1 Effects of DI3 Fractions on Proliferation and Viability of Human Leukemic HL-60 and K-562 cells and Mouse Sarcoma180 Cells

Trypan blue exclusion method was applied to study the *in vitro* effects of DI3 fractions on proliferation and viability of the cancer cells. DI3 fractions did not significantly affect the density of K-562 (Fig. 4.16) and S-180 cells (Fig. 4.17), while DI3b and DI3c reduced dose-dependently the density of HL-60 cells (Fig. 4.18). At the concentration of 200 μg/ml, DI3b and DI3c reduced the density of HL-60 cells by 50% and 39%, respectively. As shown in Table 4.8, the viability of HL-60, K-562 and S-180 were all over 98%.

4.4.2 Effects of DI3 Fractions on the Growth of Human Liver Cancer HepG2 Cells and Normal Monkey Kidney Vero Cells

MTT assay was applied to study the effects of DI3 fractions on the growth of HepG2 and Vero cells. DI3b and DI3c fractions reduced the growth of HepG2 cells in a dose-dependent manner (Fig. 4.19). At the highest concentration of 800 μg/ml, DI3b and DI3c reduced the growth of HepG2 cells by 39 % and 40 %, respectively. At 5-folds of the IC_{50} of DI3b on HL-60 cells (i.e. 1000 μg/ml), all DI3a, DI3b and

DI3c fractions did not show any significant effect on the growth of normal Vero cells (Fig. 4.20).

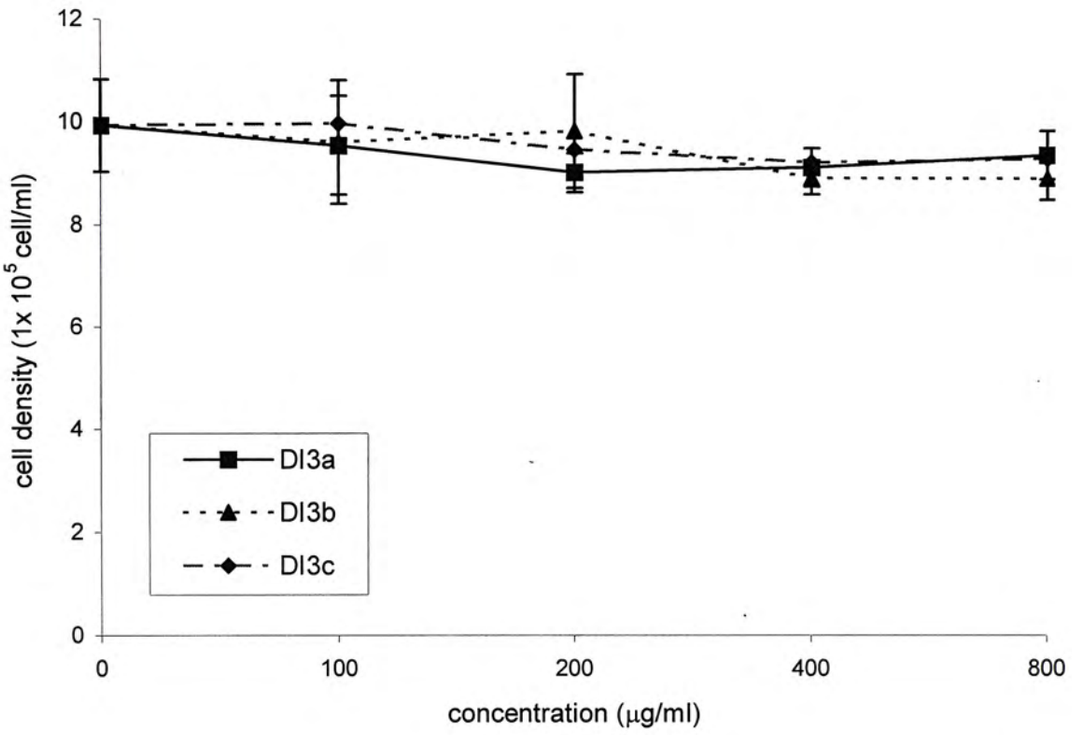

Fig. 4.16 Effect of different DI3 fractions on proliferation of K-562 cells.
Density of K-562 cells was determined by trypan blue exclusion after incubation with 100, 200, 400 and 800 μg/ml, respectivey, of different DI3 fractions for 72 h.
Results are expressed as mean ± S.D. (n=3).

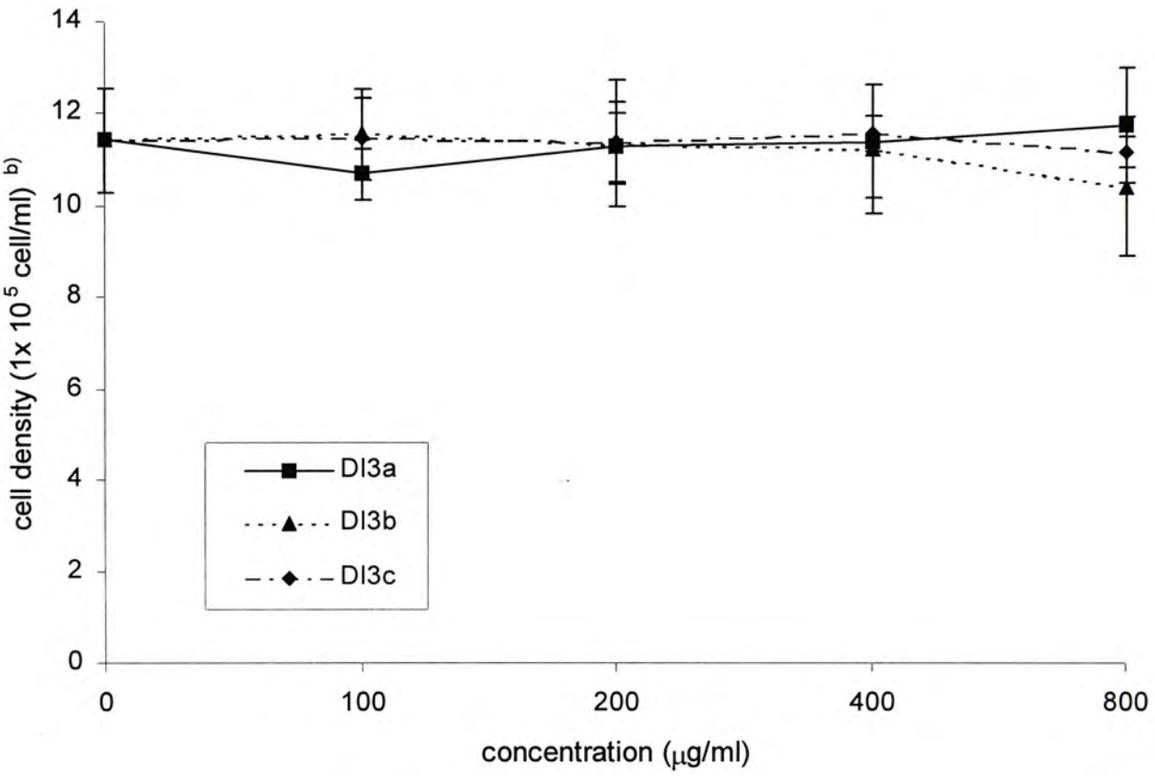

Fig. 4.17 Effect of different DI3 fractions on proliferation of S-180 cells.
Density of S-180 cells was determined by trypan blue exclusion after incubation with 100, 200, 400 and 800 μg/ml, respectively, of different DI3 fractions for 72 h.
Results are expressed as mean ± S.D. (n=3)

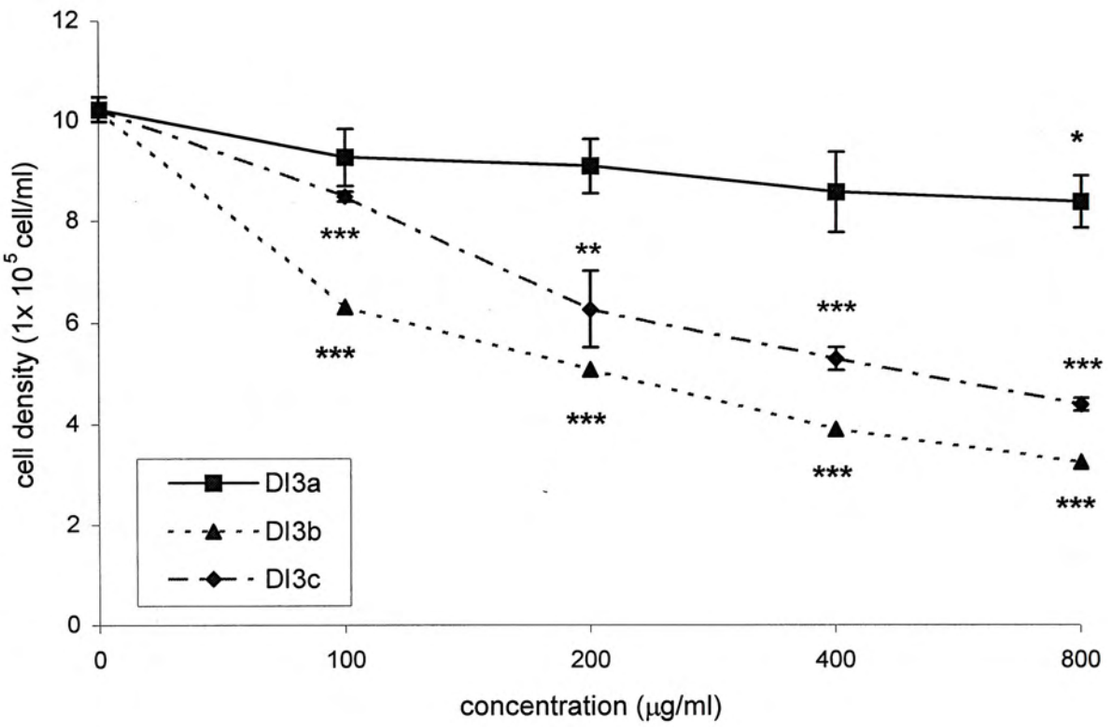

Fig. 4.18 Effect of different DI3 fractions on proliferation of HL-60 cells.
Cell density of HL-60 cells was determined by trypan blue exclusion after incubations with 100, 200, 400 and 800 μg/ml, respectively, of different DI3 fractions for 72 h.
Results are expressed as mean ± S.D. (n=3).
*, ** and ** represent statistical significance at $p< 0.05$, $p< 0.01$ and $p< 0.005$, respectively, when compared to the control level by Student's *t*-test.

Samples	Concentration	Viability (%)		
	(μg/ml)	HL-60	K-562	S-180
Control	0	99.5 ± 1.0	99.8 ± 0.3	99.6 ± 0.0
DI3a	100	98.9 ± 0.1	99.5 ± 0.5	98.9 ± 0.3
	200	99.5 ± 0.0	99.6 ± 0.3	99.3 ± 0.5
	400	99.2 ± 0.4	99.6 ± 0.7	99.5 ± 0.5
	800	99.0 ± 0.7	99.8 ± 0.3	99.9 ± 0.2
DI3b	100	98.7 ± 0.9	99.1 ± 0.8	99.6 ± 0.5
	200	98.4 ± 0.6	99.6 ± 0.3	99.4 ± 0.3
	400	97.9 ± 0.7	99.8 ± 0.3	99.7 ± 0.6
	800	98.0 ± 0.8	99.3 ± 0.3	99.8 ± 0.3
DI3c	100	99.0 ± 0.7	99.7 ± 0.3	99.7 ± 0.3
	200	99.0 ± 0.3	99.8 ± 0.3	99.9 ± 0.2
	400	98.8 ± 1.1	99.6 ± 0.6	99.6 ± 0.4
	800	98.9 ± 1.1	99.6 ± 0.7	99.9 ± 0.3

Table.4.8 Effects of different DI3 fractions on viability of HL-60, K-562 and S-180 cells after 72 h of incubation.
Results are expressed as mean ± S.D. (n=3)

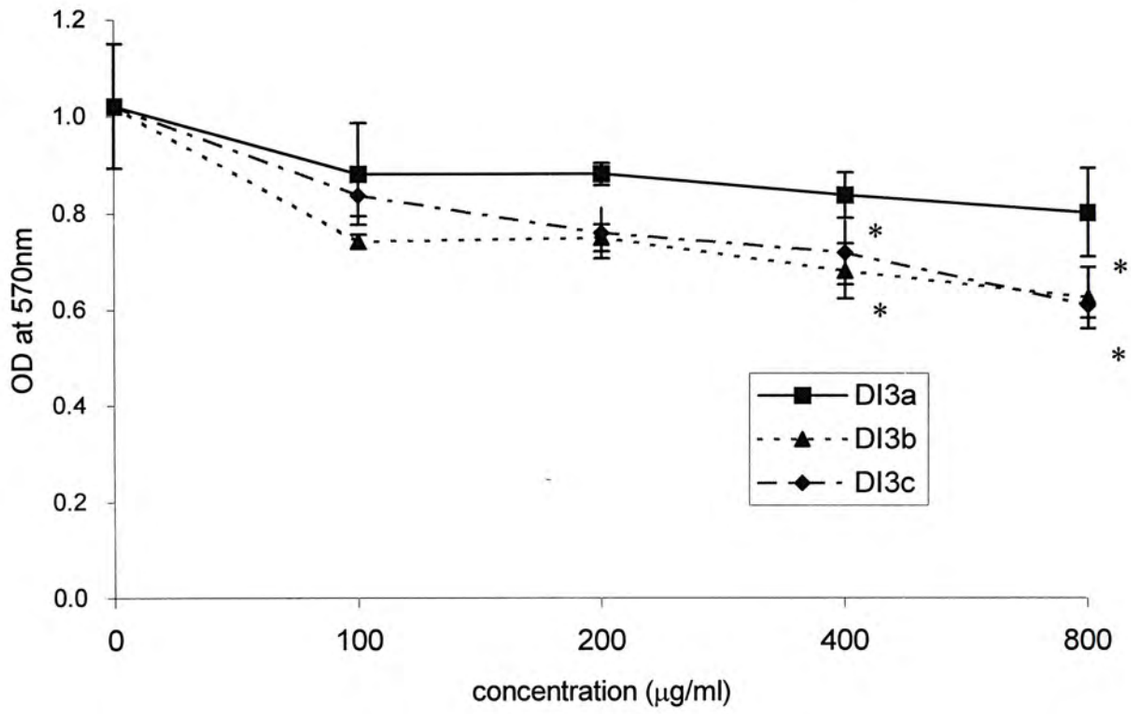

Fig. 4.19 Effects of different DI3 fractions on the growth of HepG2 cells.
The growth of HepG2 cells was measured by MTT assay after incubation with 100, 200, 400 and 800 μg/ml, respectively, of different DI3 fractions for 72 h.
Results are expressed as mean ± S.D. (n=3)
* represents statistical significance levels at $p < 0.05$ when compared to the control level by Student's *t*-test.

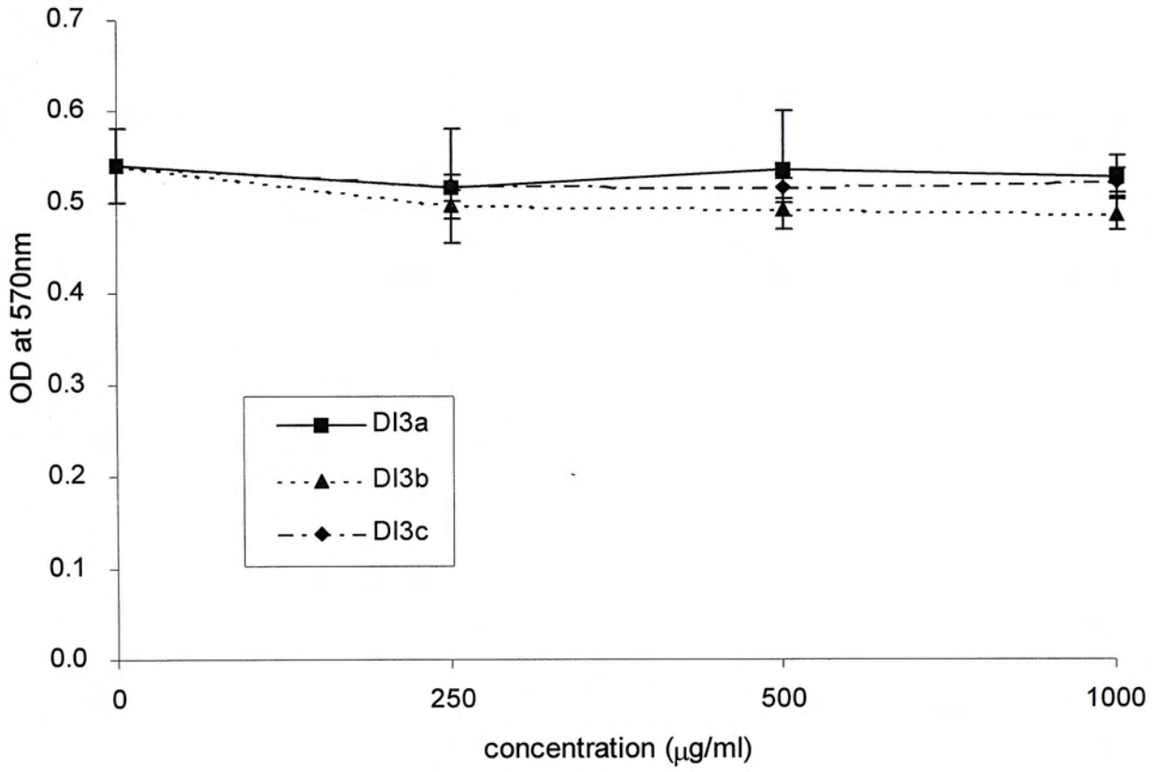

Fig. 4.20 Effects of different DI3 fractions on the growth of Vero cells.
The growth of Vero cells was determined by MTT assay after incubation with 100, 200, 400 and 800 μg/ml, respectively, of different DI3 fractions for 72 h.
Results are expressed as mean ± S.D. (n=3).

4.4.3 Effect of DI3b Fraction on Proliferation of HL-60 Cells Determined by BrdU Incorporation

To further verify the effect of DI3b fractions on the growth of cancer cells, BrdU incorporation was used to study the effect of the polysaccharides on proliferation of human leukemic HL-60 cells. As BrdU is an analogue of thymidine and its incorporation level into cellular DNA, it was used in order to detect and visualize cell proliferation at the individual cell level. After co-incubation with DI3b for 72 h, the BrdU incorporation into the cellular DNA of HL-60 cells was significantly reduced in a dose-dependent manner (Fig. 4.21). DI3b reduced the BrdU incorporation by 54% of the control level at 800 μg/ml.

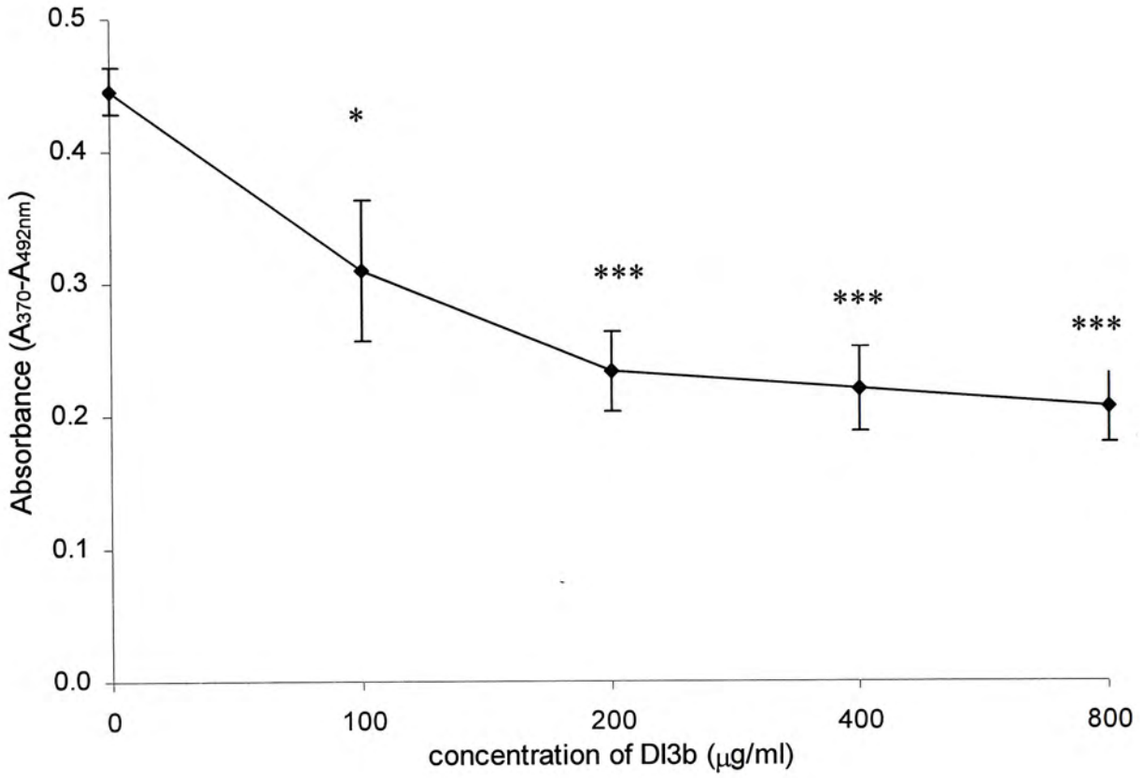

Fig. 4.21 Effects of different concentrations of DI3b fraction on BrdU incorporation in HL-60 cells.

The proliferation of HL-60 cells was indicated by the level of BrdU incorporations after incubation with 100, 200, 400 and 800 μg/ml, respectively, of DI3b fractions for 72 h.

Results are expressed as mean ± S.D. (n=3).

* and *** represent statistical significance at $p < 0.05$ and $p < 0.001$, respectively, when compared to the control level by Student *t*-test.

Chapter 5

Discussions

5.1 Extraction and Characterization of DI3 Fractions

In this study, dried fruit bodies of *Dicytophora indusiata* were subjected to hot water extraction to isolate the water-soluble components. In order to carry out thorough extraction, the process was repeated three times and each time lasted for 2-3 hours. The percentage yield of the water extracts from the dried fruit bodies was 29.67%. Since water-soluble polysaccharide-enriched fraction was commonly isolated by hot water extraction followed by precipitation with ethanol, fractional precipitations by stepwise additions of ethanol up to different concentrations were employed after the water extract was concentrated in this study (Liu *et al.*, 1996b; Wang *et al.*, 1997). The water-soluble polysaccharides were then separated into relatively higher and lower molecular weight polysaccharides by 40% and 80% ethanol precipitations, respectively. These two obtained crude polysaccharides were

designated as DI2 and DI3.

In the preliminary *in vivo* antitumor study of crude polysaccharides DI on Sarcoma 180 solid tumor, DI1 did not show any inhibitory effect; therefore it was eliminated in this study. As comparing the effect of DI2 and DI3 polysaccharides, DI3 at the dose of 100 mg/kg inhibited the growth of solid tumor significantly by 64%, which was more prominent than DI2 polysaccharides. Therefore, DI3 crude polysaccharides were chosen to undergo further bioactivity guided fractionation.

After ethanol precipitations, further separation of DI3 fraction was achieved by anion exchange chromatography. Acid polysaccharides were isolated from the neutral materials, and the separation of acid polysaccharides with different ionic strength and acidity grades was also achieved. In this study, DEAE-cellulose was used as the matrix of the chromatography. Under neutral conditions, DEAE-cellulose scarcely absorbs neutral polysaccharides, which can be eluted readily by distilled water (Scherz & Bonn, 1998). The retained polysaccharides can be eluted quantitatively with appropriate concentration of sodium chloride solution. The DI3 fraction isolated by ethanol precipitation was dissolved in distilled water, washed into the DEAE-cellulose column, and fractionated with a 0 to 1 .0 M sodium chloride solution gradient. The presence of polysaccharides was detected by phenol sulfuric assay (Dubosi *et al.*, 1956). According to the result, in addition to the peak that was

eluted by distilled water, there were two more peaks appeared and were eluted by 0.04 M and 0.5 M NaCl, respectively. DI3a fraction, which was eluted by distilled water, was composed of neutral polysaccharides, whereas both DI3b and DI3c fractions consisted of acid polysaccharides. Among the three fractions, DI3a constituted the highest proportion (69.4%) and DI3b constituted the lowest (9.2%) in the crude DI3 polysaccharides. Recovery of the three fractions from the crude DI3 polysaccharides after fractionation was satisfactory (up to about 90%).

In order to determine the polysaccharides within DI3 fraction, phenol sulfuric assay was used in this study. Phenol sulfuric assay is a reliable method that can be used to measure the sugar content in aqueous solution. Under proper conditions, such as the amount of phenol added and incubation time, the accuracy of this method can be up to 98% (Dubois *et al.*, 1956). Besides, this method is simple and the color produced is stable, which gives a definite absorption peak and can be quantified easily by a spectrophotometer.

Lowry-Folin method, instead of the Bicinchoninic acid assay (BCA), was used in this study to determine the protein content in the DI3 fractions. The Lowry-Folin method is based on both the Biuret reaction in which Cu^{2+} is reduced to Cu^{1+}, and the Folin-Ciocalteau reaction in which Cu^{1+} reacts with Folin-Ciocalteau reagent to form the final intense color. Lowry-Folin method was chosen since it is less sensitive to

the presence of reducing sugars than the BCA assay (Smith *et al.*, 1985).

The results showed that polysaccharides was the major component in DI3 fraction (>70%), but it was not the only component that was present in the three DI3 fractions. The DI3 fraction contained 12% of protein and 64%of polysaccharides in the sample. Among the three DI3 fractions, DI3a and DI3c contained the highest amounts of polysaccharides (94%) and proteins (25%), respectively.

Since higher concentrations of sodium chloride solution elute polysaccharides fractions with higher ionic strength and acidity from the DEAE-cellulose column, it is not surprised that DI3c fraction contains the highest amount of uronic acid.

Besides, as detected by gas chromatography, the monosaccharide profiles of DI3 fraction contained galactose, glucose, mannose and with trace amount of xylose. However, the ratio of monosaccharides present in DI3c is different from those present in DI3a and DI3b, respectively. The major constituent in DI3c fraction is glucose but the major constituent in both DI3a and DI3b fractions is mannose.

As shown by HPLC study, DI3c fraction was a relatively homogenous polysaccharide. The average molecular weight of DI3c polysaccharide, which was determined by measuring the retention times of the peak and compared it to the dextran standard, is 3.3 kDa. DI3b and DI3c fractions, on the other hand, contained two and three groups of polysaccharides, respectively.

With references to the findings of the previous studies, many of the antitumor mushroom polysaccharides are protein-containing polysaccharides, which are similar as that in DI3c fraction. The protein contents within the polysaccharides from mushroom *Tricholoma mongolicum*, *Pleutrotus sajor-caju* and *Coriolus versicolor* range from about 11% to 24%, whereas that in the fraction of DI3 ranges 3-25% (Zhuang *et al.*, 1993; Tsukagoshi *et al.*, 1994; Wang *et al.*, 1996a).

Moreover, the main constituent of other reported polysaccharide T-2-HN and T-3-M from *Dictyophora indusiata*, which are O-acetylated (1→3)-α-D-mannan and α-(1→3)-linked D-mannan, is mannose. It was found DI3a and DI3b polysaccharides fractions, like T-2HN and T-3-M, are also mainly composed of mannose (Hara *et al.*, 1982a; Hara *et al.*, 1988).

Besides, as compared the molecular weight of the DI3 fractions with the polysaccharides extracted from *Dictyophora indusiata* by Hara *et al.* and Ukai *et al.* (stated in Table 2.1), the average molecular weight of DI3c fraction is much smaller (~ 3.3 kDa) than those extracted by Hara *et al.* and Ukai *et al.* (>10 kDa).

5.2 Antitumor Effects of *Dictyophora indusiata* Polysaccharides

In order to investigate the *in vivo* antitumor effects of *Dictyophora indusiata* polysaccharides, 7-8 weeks old male BALB/c mice were used in this study. BALB/c mice were commonly used in many other studies, and the use of male mice is to get rid of the influence of hormonal changes in female mice. Besides, the mice used were acclimatized for a week before starting of each experiment, so as to minimize the variation of mice within the group. After inoculation of S-180 cells into the back of the mice, they were left for 3 days before i.p injection of the sample. The reason for that is not only to allow time for the development of solid tumor, but also to mimic the situation that treatments always received after the occurrence of tumor.

According to the results, DI3 was more effective than DI2 in inhibiting the growth of Sarcoma-180 tumor, which gave an inhibitory rate of 64%. DI3 was further fractionated into three fractions: DI3a, DI3b and DI3c.

Among the three polysaccharides fractions of DI3, DI3c was the most potent fraction in inhibiting the growth of S-180 tumor, however, the antitumor effect of DI3a fraction was not significant. It was not surprised that the neutral polysaccharides fraction of DI3 exhibited lesser antitumor activity, since the neutral polysaccharides from *Tricholoma* sp. (strain STC20-T1) also exhibited the least antitumor activity when compared to other polysaccharides fractions (Liu *et al.*,

1995)

In order to compare the antitumor effects of DI3 polysaccharides before and after fractionation in DEAE-cellulose column, DI3 crude polysaccharides and DI3c fraction at different doses were administered to the tumor-bearing mice. Both the two fractions possess antitumor activity dose-dependently. This is in agreement with the previous reports, that CF-1 and CF-4 of *Griofola frondosa* polysaccharides and GL-B of *Ganoderma lucidum* polysaccharides also inhibited the growth of tumors dose-dependently (Ohno *et al.*, 1984; Zhang & Lin, 1999). Furthermore, the minimal doses of the crude DI3 polysaccharides and fractionated DI3 fractions that significantly inhibit the tumor growth were found to be 25 mg/kg and 12.5 mg/kg, respectively. DI3c fraction was found to posses the most effective antitumor activity in the present study. However, the anticancer activity was relatively less effective when compared to some of the mushroom polysaccharides administered at similar doses or even at lower dose. Schizophyllan has been found to induce complete regression of Sarcoma 180 solid tumor in Swiss mice treated with a dose of 10 mg/kg for 10 consecutive days (Komatsu *et al.*, 1969). Lentinan also induced complete regression of S-180 tumor in ICR mice treated with a dose of 1 mg/kg for 10 consecutive days (Chihara *et al.*, 1970). The difference in effectiveness may be due to the uses of different strains of mice. Further studies are required to demonstrate

the antitumor effect of DI3c in other strains of mice.

Although the extraction method used in this study was not targeted on the isolation of glucan from mushroom *Dictyophora indusiata*, which was not the same as that in the isolation of PSK (Krestin) from mushroom *Coriolus versicolor,* the result showed that the proteins amount and monosaccharides profile of DI3c fraction was very similar to that of PSK. PSK (Krestin), which is used clinically in anticancer treatment, is a β-D-glucan complex containing 25-38% protein. It was mainly made up by glucose with small amount of mannose, fucose, xylose and galactose (Tsukagoshi *et al.*, 1984). In DI3c fraction, 25% of its weight was protein residue, and the major constituent monosaccharide is glucose with small amount of mannose, galactose and xylose, Therefore, DI3c fractions may be an antitumor glucan linked with heterosaccharide chains of galactose and mannose and it may not be neglected the possibility that the protein part of DI3c fraction may also participate in the antitumor activity on tumor-bearing mice. Further studies are demanded to compare the antitumor activities of the polysaccharopeptides, polysaccharides and proteins components of the DI3c fraction.

Since there were no significant changes in body weight of the DI3c-treated group as compared to that of the normal weight control, it manifested that administration of DI3c did not affect the increase in body weight. Besides, no death

of mice was observed either in control or treatment group throughout the experiments. Accordingly, DI3c fraction may not be thought to display antitumor activity through direct toxicity action against tumor cells.

Cytokines system is a key-contributing factor in the immune system and inflammatory responses (Kelso, 1998). Many of the mushroom polysaccharides that show antitumor responses have been found to associate with immunomodulating cytokine production *in vivo,* such as the two well-known medicinal mushroom polysaccharides, schizophyllan and PSK (Ooi & Liu, 1999).

To gain insight into the antitumor activity by DI3c fraction, sandwich type enzyme-linked immunosorbent assay (ELISA) was used to study the modulatory activities of DI3c fraction on cytokine production. This assay method is highly sensitive and can detect the presence of cytokines, but also can determine the cytokine production at very minute quantity (pg/ml) (Caponi and Migliorini, 1999). Previous studies have shown that mushroom polysaccharides and proteins modulate TNF- α and IL-2 productions *in vivo*. PSK and GLP increased the expression level of TNF-α in peritoneal exudates cells of mice (Liu *et al.*, 1996b; Hsu *et al.*, 1997). And Fip-vvo, a protein purified from mushroom *Volvariella volvacea*, was shown to elevate the expression level of IL-2 in splenocytes of mice (Ooi *et al.*, 2002). In this study, the immunomodulatory effect of DI3c fraction on TNF-α and IL-2 production

in BALB/c mice was investigated. Administration of 12.5 mg/kg DI3c fractions for 5 consecutive days produced an elevated level of TNF-α. LPS administration in normal mice further elevated the TNF-α production. However, the concentration of IL-2 in blood sera of mice did not shown any significant changes. Bacterial lipopolysaccharides (LPS), which responsible for immune response, can activate macrophages to release cytokines including TNF-α, IL-1 and IL-6 (Nathan, 1987). Therefore, the priming effect of TNF-α production by DI3c fraction is strongly related to the activation of macrophages. Since TNF-α possess direct cytotoxicity on tumor cells via apoptosis and necrosis, and the TNF-α production would further activate the macrophage to produce more TNF-α (Brophy, 2001), the inducing effect of TNF-α demonstrated the antitumor action of DI3c fraction when it was administered in tumor-bearing or normal mice. As reported by Ohno *et al.* (1995), a (1→3)-β-glucan from *Grifola frondosa* (GRN) also showed the priming effect of TNF-α production in ICR, MRL/1 pr as well as BALB/c mice (Ohno *et al.*, 1995).

Apart from *in vivo* antitumor study, four different cancer cell lines, including human leukemic HL-60 and K-562 cells, human liver cancer HepG2 cells and mouse Sarcoma 180 cells, were used to investigate the antiproliferation and cytotoxic effects *in vitro*. In order to study the growth of different cancer and normal cell lines after incubation with different DI3 fractions, either trypan blue exclusion method or MTT

reduction assay were used. The growth of HL-60, K-562, S-180 and HepG2 cancer cells were not inhibited by DI3a fraction. Since DI3a fraction contains mainly of neutral polysaccharides, it implies that neutral polysaccharides may not have antitumor activities.

DI3b and DI3c fractions were able to inhibit the proliferation of HL-60 and HepG2 cells dose-dependently. Although the efficacies of DI3b and DI3c on reducing the growth of HepG2 cells are about the same, DI3b was a more effective in inhibiting the proliferation on HL-60 cells when compared to DI3c. DI3b at 800 μg/ml inhibited the growth of HL-60 cells by 68%, while only 57% of inhibition was observed with DI3c fraction. It is rare that mushroom polysaccharides inhibit the proliferation of cancer cell directly. Polysaccharides from *Ganoderma lucidum* MGLP1 did not show inhibition of HL-60 proliferation up to 750 μg/ml. However apoptosis was observed after the cells have been incubated with the conditioned medium from MGLP1 activated splenocytes (MGLP-S-CM) (Hu and Lin, 1999). Therefore, it was interesting that both DI3b and DI3c could inhibit the proliferation of HL-60 *in vitro*.

In a further study, BrdU incorporation in HL-60 cells was measured after incubation with different concentrations of DI3b fraction to verify the effect of DI3b fraction on proliferation of HL-60 cells. The BrdU incorporation was decreased by

54% of the control level after incubation with DI3b fraction at 800 μg/ml for 72 h. Since BrdU is an analogue of thymidine and its incorporation level into the cellular DNA is a measure of proliferation, the decrease in BrdU incorporation indicated inhibition of HL-60 cell proliferation by DI3b fraction. In the previous studies, this method has also been utilized by Huong *et al.* (1991) and Magaud *et al.* (1988), respectively, to measure the proliferation of human lymphocyte. Both trypan blue exclusion and BrdU incoporation assays showed that DI3b fraction inhibited the proliferation of HL-60 cells dose-dependently.

DI3 fractions did not retard the growth of Vero cells even when the dose was up to 5-folds of the IC_{50} of DI3b fractions on HL-60 cells. The viability of HL-60, K-562 and S-180 were all over 98%. All these results imply that the antiproliferative action of DI3 fractions on HL-60 cells may be modulated by cell cycle regulation (Kawamura *et al.*, 2000), rather than by inducing apoptosis or necrosis (direct cytotoxicity).

According to the previous *in vivo* study, the DI3c fraction showed inhibition of S-180 cells at a minimal dose of 12.5mg/kg. However, DI3 fractions did not show any antiproliferative effect toward S-180 tumor cell line *in vitro*. Moreover, DI3 fractions did not show direct cytotoxic effects on Vero cells. All these results support the hypothesis that the antitumor effect of DI3c fraction on S-180 is potentiated by

modulating the immune system of the host.

5.3 Further Studies

In this study, it was found that DI3c is the most prominent fraction in inhibiting the growth of S-180 in tumor-bearing mice, and the possible mechanism of antitumor activity may be due to the elevated TNF-α level in blood serum. However, this study only targets on two of the cytokines, namely TNF-α and IL-2. As in the study by Liu *et al.* (1999), up-regulation of gene expression of IL-1α, IL-1β, TNF-α, IFN-γ and M-CSF by PSPC and lentinan, respectively, were detected in mice, other cytokines such as interferons or different types of interleukin, can also been chosen for further study (Liu *et al.*, 1999). Since DI3c is a relatively homogenous fraction and is composed of small molecular weight polysaccharides (3.3 kDa) with high water solubility, it is worth to develop this fraction as an antitumor injectant. Furthermore, since different dosage and timing of administration may also alter the efficiency (Wang and Zheng, 1997), more studies are required to investigate the optimal administration schedule of DI3c.

Apart from the *in vivo* antitumor effect of DI3c, DI3b is also a potent fraction in inhibiting the proliferation of HL-60 and HepG2 cancer cells. Although the antiproliferative effect of DI3b is prominent on these two cancer cells, especially on HL-60 cells, the impurity in DI3b may affect the antitumor efficacy of this fraction. Therefore, further fractionation and purification of this DI3b fraction in Sepharose or

Sephadex column is required to obtain relatively more purified compounds for further investigation.

Since, the antiproliferative activity of DI3b fraction may be due to the cell cycle regulation, the mechanism of DI3b in inhibiting the proliferation of cancer cells can be studied in more detail. With the aid of flow cytometry or by using western blot analysis, the status or the level of the cell cycle regulatory proteins, for examples the cyclins, cyclin dependent kinase inhibitor and tumor suppressor proteins, can be investigated.

Chapter 6

Conclusion

In the present study, three mushroom polysaccharide fractions have been isolated from the fruit bodies of edible mushroom, *Dictyophora indusiata*, which are designated as DI3a, DI3b and DI3c, respectively.

All of the three fractions have been subjected to *in vivo* and *in vitro* antitumor studies. DI3c fraction, which comprises of polysaccharopeptide of 3.3 kDa and contains 25% of proteins, is the most prominent fraction in inhibiting the growth of S-180 in tumor-bearing mice. The antitumor effect of DI3c fraction is dose-dependent and at dosage of 50 mg/kg, the inhibition rate was up to 76%. DI3b fraction, which consists of three groups of polysaccharides, is the most prominent fraction in inhibiting the proliferation of cancer cells *in vitro*. DI3b inhibits the proliferation of HL-60 and HepG2 cells dose-dependently and the IC_{50} of DI3b

fraction on HL-60 cells was 200 μg/ml.

DI3c elevates serum TNF-α level showing that the *in vivo* antitumor effect of DI3c may be mediated by TNF-α induction, which is cytotoxic on the cancer cells via apoptosis and necrosis. The priming effect of DI3c is related to the activation of macrophage.

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